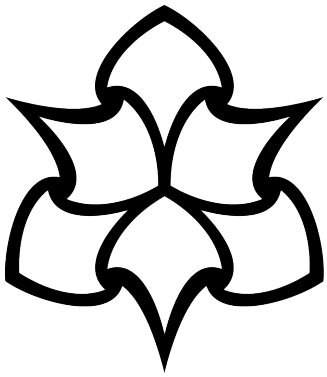

Novel Thienopyridines as

Anti-platelet agents

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Abstract

Platelets have well-established roles in haemostasis, and this requires a fine balance to maintain platelets in a resting state in the absence of vessel injury and allow rapid activation upon stimulation. Platelet hyperactivity is associated with several serious health conditions such as Acute Coronary Syndrome (ACS), Venous thromboembolism (VTE) Myocardial Infarction and Stroke, and so anti-platelet therapies are a critical tool in the management of such diseases.

This thesis evaluated the use of six thienopyridine compounds to modulate platelet activity. Members of the thienopyridine family are currently in clinical use in the UK but the variable response to specific drugs and the associated side effects warrant continued identification and refinement of alternative compounds.

The work demonstrated that these six novel thienopyridine derivatives were able to inhibit ADP-induced platelet activation (assessed by CD62P expression, PAC1 binding and platelet-leukocyte aggregate formation) and aggregation (assessed by light-transmission aggregometry) in platelets obtained from healthy participants. Furthermore, the compounds seemed to show enhanced activity when compared with the standard thienopyridine, clopidogrel (active metabolite). The signalling pathways affected by these compounds were studied and found that Ca^{2+} flux was affected in ADP-stimulated platelets and VASP phosphorylation was maintained. The effects on PLCγ2, cPLA2 and PI3k/Akt were variable and inconsistent.

As endothelial cells also have a crucial role in haemostasis and prevention of inappropriate clot formation, and have been shown to be responsive to ADP, this thesis also evaluated the effect of the compounds on endothelial cells adhesion molecule expression. All six compounds were shown to inhibit CD62P, CD62E and VCAM-1 in stimulated HUVEC cells, suggesting a new area for drug development.

Finally, the thesis shifted focus on to the role of platelets in immunological responses. Since platelets have been reported to have central roles in cancer development and to sequester proteins from within the microenvironment, the final part of the work looked at whether platelet function can be affected by the tumour cell secretome and attempted to characterise the cytokines responsible for the effects. The results showed that the tumour cell secretome can inhibit platelet activation and aggregation and provides a mechanism by which to explain increased bleeding tendency in cancer patients.

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List of Abbreviation

5-HT	5-hydroxytryptapmin
AA	Archidonic Acid
Ab	Antibody
Ag	Antigen
ADP	Adenosine diphosphate
AHA	American Heart Association
ASA	Acetylsalicylic acid
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CK-MI	Creatin kinase myocardial infraction
COX-1	Cyclooxygenase-1
cPKC	Classical protein kinase C
CPTP	Cyclo-pentyl-trizol-pyrimidin
Ct	Closure time
bFGF	Basic fibroblast growth factor
DAG	Diacyglycerol
DAPI	Diamidino-2-phenylindol
DCP_2Y_{12}	dentric cells P_2Y_{12}
DTS	Dense tubular system
ECGS	Endothelial cell growth supplement
ERK	Extracellular-signal regulated kinase
FII	Prothrombin
FBS	Fetal bovin serum

FC	Fragment Crystallisable
FcRγ	Fc Receptor γ chain
GPCR	G proteins couple receptor
GPIb-IX	glycoprotein Ib-IX-V complex
GPVI	glycoprotein VI
GSK3	Glycogen synthase kinase-3
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
IL-1	Interleukin-1
IL-6	Interleukin-6
IP_3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
LAT	Linker for activated T-cell
LTA	Light transmission aggregometry
MMPs	matrix metalloproteinase
MEK	Mitogen activated protein kinase APK/Extracellular-signal regulated kinase
NO	Nitric oxide
NOS-III	Nitric oxide synthase III
nPKC	Novel protein kinase C
P2Y12	Purinergic receptors
PAF	Platelets activating factor
PAI-1	Plasminogen activator inhibitor -1

PAR-1	Protease activated receptors -1
PAR-4	Protease activated receptors -4
PDGF	platelet derived growth factor
PDK-1	Phosphoinositide-dependent kinase -1
PF4	platelets factor 4
PFA-100	Platelet function analyzer-100
PGE2	Prostagladin E2
PGG2	Prostagladin G2
PGH	Prostagladin H synthase
PGH2	Prostagladin H2
PGI2	Prostagladin I2
PKC	Protien kinase C
PLC	Phospholipase C
PLCβ	Phospholipase C beta
PLCγ2	Phospholipase C gamma 2
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-diphosphate
PIP_3	phosphatidylinositol 3,4,5-triphosphate
PSGL-1	P-selectin glycoprotein ligan-1
RACK	Receptor for activated C-kinase
RANTES	Regulated on activation normal T expressed and secreted
RIPA	Radioimmunoprecipitation assay buffer
RSC	ReeD-Sternberg cells
SCCS	surface connected canncular system

SFKs	Ser family kinase
SH	Scr homology
SLP-76	Leukocyte phosphoprteins 76 kDa
Syk	Tyrosine kinase
TBST	Tris buffer salin with Tween 20.
TEMED	N, N,N,N-Tetramethylenediamine
TF	Tissue fctor
TFPI	Tissue factor pathway inhibitor
TGF-β	Transforming growth factor –β
TLR	Toll-like receptor
TNF-a	Tumour Necrosis Factor –a
TP	Thromboxane receptor
tPA	Tissue plasminogen activator
tPKC	Typical protein kinase C
TSP-1	Thrombospodin-1
TxA2	Thromboxane A2
UA	Unstable angina
UTP	Uridin triphosphate
VEGF	Vascular endothelial growth factor
VWF	Von Willbrand factor

Chapter 1: Introduction

Chapter 1 : Introduction

Platelets have a well-established role in haemostasis and thrombus formation (Dorsam and Kunapuil, 2004). Although this process is tightly regulated, hyperactivity/heightened sensitivity of platelets, as well as hypoactivity can occur, and results in inappropriate thrombus formation or bleeding tendency respectively (Key *et al.*, 2009). Hyperactivity of platelets contributes to myocardial infarction and stroke, two of the most common causes of morbidity in the developed world (Zetterberg and Svensson, 2016). The ability to control platelet activity and reduce adverse thrombus formation is therefore a critical tool in modern clinical practice.

1.1 Platelet biology

Platelets are enucleated cellular fragments that play an important role in thrombosis and haemostasis. These cells are produced by fragmentation of the megakaryocyte cytoplasm, where each megakaryocyte produces approximately 1000-5000 platelets (Hoffbrand et al., 2011). The normal circulating concentration of platelets in healthy individuals is 150-440 ×109/L (McKenzie, 1996). Platelets normally flow in the blood in a dormant (inactive) state but respond rapidly to vessel damage by activation and aggregation at the site of injury in order to stop bleeding. Platelets express a number of cell surface receptors and contain a vast array of proteins that allow them to achieve such quick activation and aggregation. Changes to the expression of these receptors or intercellular proteins may lead to a hypo- or hyper-active state resulting in excessive bleeding or inappropriate thrombosis respectively (McKenzie, 1996; Hoffbrand et al., 2006).

1.2 Platelet structure

Platelets have a discoid shape and are extremely small in comparison to red blood cells, with a diameter of 2-3μm (Fig 1.1). The platelet membrane is complex and constitutes proteins and lipids, both of which are important in platelet haemostatic function (Fritsma, 2015). The protein component of the platelet membrane comprises the Glycocalyx that coats the platelet surface and is essential for platelet extracellular signaling (Hoffbrand et al., 2011). The glycocalyx consists of different glycoproteins such as glycoproteins Ia (GPIa) that bind collagen, glycoprotein Ib and IIb/IIIa ($\alpha_{IIb,}\beta_3$) which bind Von Willbrand factor (VWF) and $\alpha IIb_{,}\beta_{3,}$ which is a specific receptor for fibrinogen. The middle layer of the membrane is composed of phospholipid, which forms the basic structure of platelets, and supports platelet activation by supplying arachidonic acid. It has a role in the activation of coagulation factor X and Prothrombin (FII). The inner layer of the plasma membrane invaginates into the platelet and translates signals from the surface to the platelet's sub-structure through the surface connected cannicular system (SCCS), allowing activation of the platelet following appropriate stimuli. The Dense tubular system (DTS) works in parallel to SCCS sequestering Ca^{2+} and some enzymes that are involved in platelet activation such as phospholipase A_2, Cyclooxygenase and TXA2 synthesis (Gresele et al., 2012; 'Platelet Structure and Function', 2015).

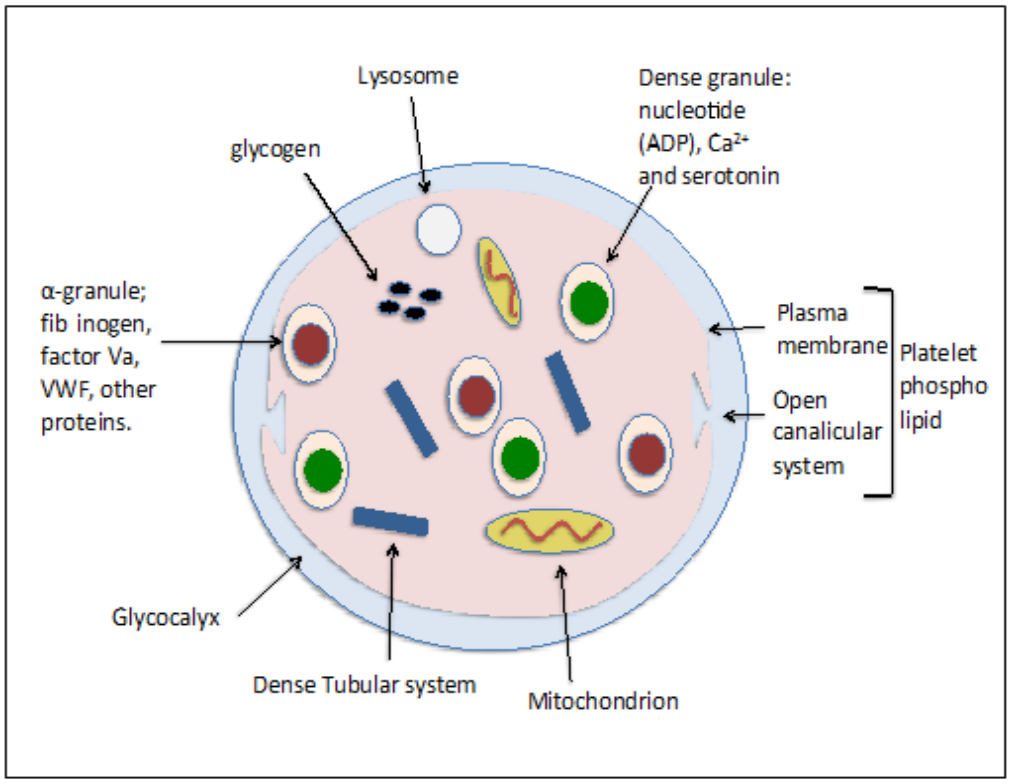


Figure 1.1 Platelet Structure. Platelets comprise membranous components (plasma membrane, surface-connected canalicular system, and dense tubular system), mitochondria, microtubules, and glycogen, in addition to storage organelles: α-granules, dense bodies and lysosomes (Adapted from, Hoffbrand et al., 2011).

1.2.1 Platelet granules

There are three different types of storage granules present in platelets; α-, dense- and lysosome-granules (Hoffbrand et al., 2011; Ware et al., 2013). Recently presence of a fourth granule type has been proposed, referred to as the T-granule (Morrell et al., 2014; Thon et al., 2012). The characteristics of the granules are depicted in the Table 1.1

Table 1-1 Characteristics of platelet granules. Table complied from data from (Hoffbrand et al., 2011; Morrell et al., 2014)

Granule	**Number per platelet**	**Size (nm)**	**Contents**	
Alpha	50-80	200-400	Heparin agonist, platelet factor 4 (PF4), platelet derived growth factor (PDGF), β-thromboglobulin, fibrinogen, von willebrand factor (VWF), coagulation factors	Promote vascular repair
Dense	2-7	150	Adenosine Diphosphate (ADP), Adenosine Triphosphate (ATP), Ca^{2+}, 5-hydroxytryptapmin	Recruitment of additional platelets to site of injury. Consequently, platelet shape change and platelet aggregate.
Lysosome granules	1-3	Unknown	Glycohydrolase enzymes and catalase	Degrade the material that engulf by phagocytosis.
T-granules	Unknown	Unknown	toll-like receptor (TLR)	Platelet recognition of invading microbes

1.3 Platelet activation

Platelets are Ca^{2+}normally in a resting state when in the peripheral circulation but they become activated at the site of vascular damage following exposure to adhesive proteins on endothelial cells, such as collagen and VWF, or by triggering from other platelet agonists such as ADP, Thrombin or TXA2 (Li et al., 2010). Upon binding of the agonist to the platelet and consequent platelet activation, platelets reorganize the cytoskeleton to change from a

discoid shape to increase the surface area for adhesion to the immobilized surface. This leads to a change in morphology, extending filopodia and eventually lamelliopodia which play a role in strengthening the adhesion to injured vessels and other platelets (Aslan et al., 2012). Moreover, platelet granules also release their contents into the microenvironment to exacerbate platelet aggregation and adhesion (Fritsma, 2015).

1.4 Platelets Agonist receptors

Platelets have an array of receptors on their surface, which can be categorized in to adhesion receptors, G-Protein Coupled receptors (GPCR) and Immunoreceptor tyrosin-based activation motif (ITAM) receptors.

Collagen and VWF are exposed on the sub-endothelium at the site of the damaged vessel wall (Fig. 1.2) (Clemetson, 2011). VWF captures fast flowing platelets at the site of injury and binds to GPI-V-IX receptors on platelets under high shear conditions (Bergmeier et al., 2006; Li et al., 2010; Bye et al., 2016). This complex of VWF: GPI-V-IX also induces platelet activation and plays a role in stable platelet adhesion and aggregation (Bye et al., 2016). VWF also facilitates the interaction between a captured platelet and collagen where it binds to α2β1 and GPVI on the platelet surface, which leads to stable adhesion, initiation and activation of platelet signalling (Clemetson, 2011; Bye et al., 2016). Once platelets are activated via collagen or VWF, they synthesise secondary mediators including TXA2 via cyclooxygenase-1 activity and release ADP from their dense granules. These mediators amplify platelet responses and activate the coagulation pathway (Clemetson, 2011). ADP binds to P2Y1 and P2Y12 receptors, while TXA2 interacts with TP receptors (Fig. 1.2). Activation of P2Y1 and TP receptors leads to an increase in intracellular Ca^{2+} levels, while

inhibiting adenyl cyclase, causing suppression of the formation of cyclic adeonosin momophosphate (cAMP) (Li et al., 2010). Consequently, platelet activation and responsiveness increase due to these changes.

One of the important proteins at the site of injury that is involved in adhesion is tissue factor (TF). TF plays a crucial role in platelet activation of the coagulation cascade and the end point formation of thrombin from prothrombin (Clemetson, 2011). Thrombin itself is a platelet activator and stimulator of platelet aggregation through protease activated receptor (PAR-1 and PAR-4) on the platelet surface. In addition, thrombin plays a role in the stabilizing thrombus by activation of fibrinogen to fibrin mesh (Bye et al., 2016).

1.5 Platelet signalling.

Platelet signalling involves a complex network of G-protein-coupled receptors, numerous G-proteins and downstream protein kinases, all of which act to stimulate Ca^{2+} signalling which leads to platelet shape change, release of platelet granule contents and change in expression of adhesion molecules (Stalker et al. 2012). The central regulators include various phospholipase C isoforms (including PLCβ and PLCγ2), protein kinase C (PKC) and phosphatidylinositide-3-kinase (PI3K) (Fig. 1.2) (Bye et al., 2016; Stalker et al., 2012).

The principal signaling events that occur following stimulation with the main platelet agonists ADP, collagen, thrombin and thromboxane are shown in Figure 1.2. Although it was at one time assumed that there were distinct signaling pathways that became activated in response to a given agonist, evidence demonstrates the extensive crosstalk and interplay that occurs to ultimately produce a platelet response (Stalker et al. 2012).

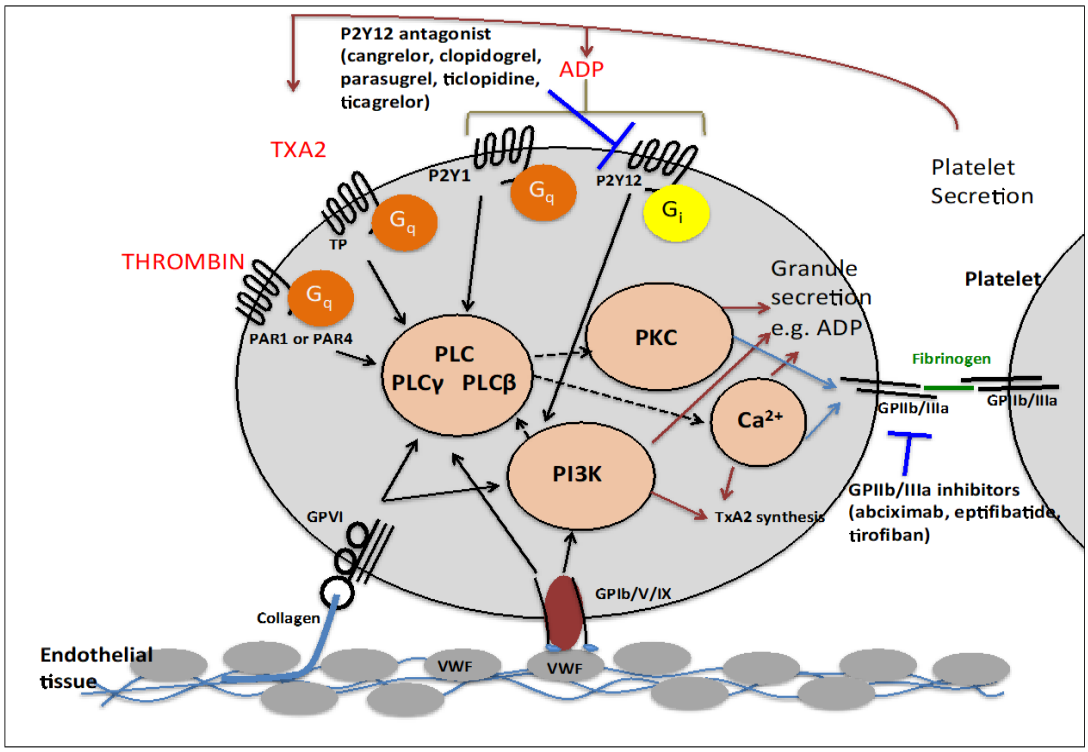


Figure 1.2 Stimulation of platelets by various agonists and their major signalling events. The key regulatory components of signalling involve PLC, PI3K and PKC which lead to platelet secretion of secondary mediators (ADP and TxA2) that work as positive feedback in platelet activation and amplify the aggregation. P2Y12 antagonists and GPIIb/IIIa work against these receptors to supress their

1.5.1 Platelet signalling via PLC isoforms.

It has been shown that platelets without PLC cannot be stimulated nor show a Ca^{2+} flux in response to an agonist (Yang et al., 1996). PLC has two isoforms, PLC-γ2 and PLC-β, with distinct downstream signaling events. Activation of PLCβ occurs by Gq-coupled GPCR such as TP (TXA2's receptor), P2Y1 (ADP's receptor) and PAR-1 and PAR-4 (thrombin's receptors). It is important in platelet signaling and is involved in secretion of secondary mediators from platelet granules such as ADP and TXA2. PLCβ catalyses the reaction of phosphatidylinositol 4,5biphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG leads to activation of PKC, and IP_3 activates the Ca^{2+} flux into the platelet cytosol by binding of IP_3 to its receptor on the dense tubular system (DTS) (Szumilo

and Rahden-Staron, 2008; Williams, 1999; Bye et al., 2016). Deleting the Gq gene in mice was shown to prevent activation of PLCβ leading to platelets unresponsive to thrombin, ADP and TXA2. Consequently, inactivated PLCβ lead to impaired, Ca^{2+} release and PKC activation (Nonne et al., 2005).

PLC-γ2 activation is widely associated with the interaction of VWF and GPIb-IX-V, or collagen with GPVI. GPVI is one immunoglobulin superfamily that is a platelet specific receptor, bearing an ITAM fragment crystallisable (Fc) γ-chain receptor (Nieswandt and Watson, 2003). The signaling downstream of PLC-γ2 is complex and requires contribution from a number of tyrosine kinases including linker activated T cell (LAT), which itself is activated by Syk, which is downstream of Scr family kinase (Bye et al., 2016). PLC-γ2 activation requires a binding SLP76 with LAT through DAG, to allow Btk to allow phosphorylation of PLC-γ2 (Bye et al., 2016). The activation of PLC- γ2 (and other tyrosine kinases) is summarised in Fig 1.3.

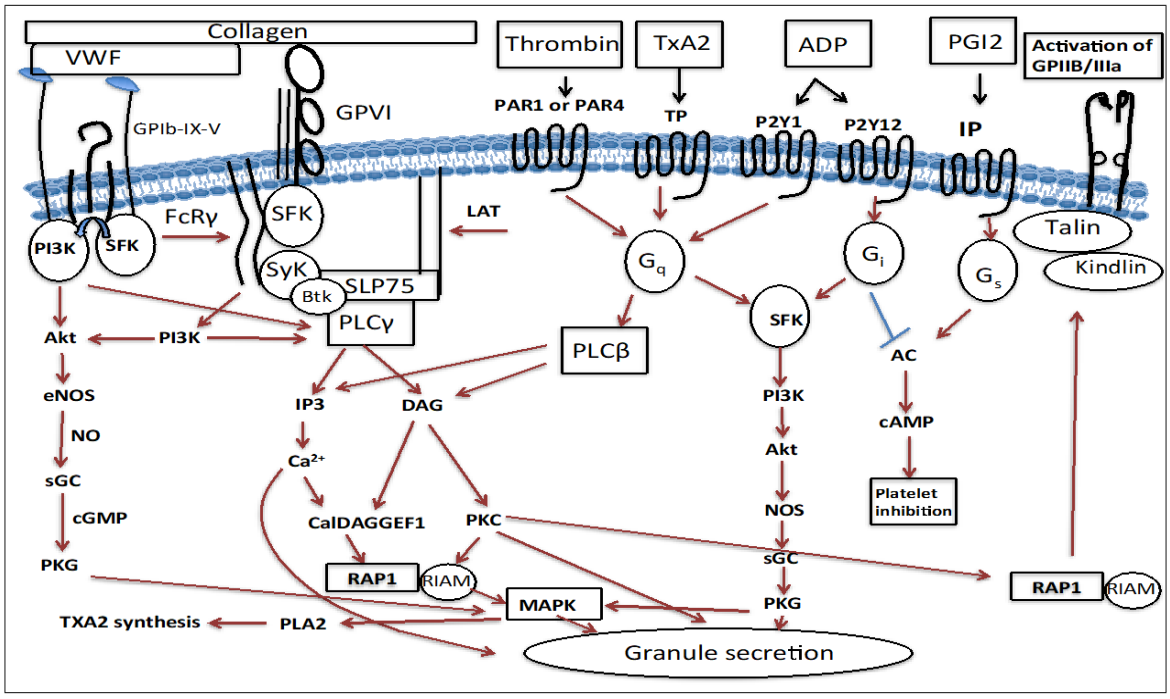


Figure 1.3 Detailed signaling events following stimulation with various agonists (adapted from Li et al, 2010).

The binding of collagen and fibrinogen to their respective integrins α2β1 and αIIbβ3 can also activate PLC-γ2, and presence of LAT in this pathway is not required (Asselin et al., 1997; Z. Y. Li et al., 2010; Bye et al., 2016). A third pathway of activating PLC-γ2 is by the binding of ADP to P2Y12, which in turn results in Gi-PI3K-Rab1b complexing and plays crucial role in platelet activation (Nonne et al., 2005). To highlight the crucial role of this phospholipase in platelet activation, deletion of PLC-γ2 in a mouse model was shown to abolish thrombus formation upon vessel injury (Nonne et al., 2005).

1.5.3 Platelet signalling via PKC.

PKC consists of a family of isoforms that play crucial roles in platelet intracellular signaling. These isoforms have been divided into three different kinds according to their mechanism

and structure. Classical PKC (cPKC) which requires DAG and Ca^{2+} for its activation, novel PKC (nPKC) which is activated by DAG without need for Ca^{2+} and the third type is typical PKC (tPKC) which is insensitive to DAG and Ca^{2+} for activation. There is still some controversy surrounding some PKC isoforms in platelet activation (Z. Li et al., 2010).

PKCδ is a novel PKC important in platelets, where it acts to trigger activation of MEK/ERK and p38 MAPK signalling. This leads to the subsequent release of thromboxane A2, which is essential for collagen-induced, but not thrombin-induced platelet activation and aggregation (Yacoub et al., 2006). A broad spectrum of studies that use PKC inhibitors or mice deficient in individual PKC isoforms, have demonstrated that PKC isoforms have positive effects on platelet granule secretion, integrin activation, synthesis of TXA2, aggregation and thrombus formation (Harper and Poole, 2010). However, some isoforms of PKC appear to have negative effects on platelet activation, but this concept is controversial (Nishikawa et al., 1980; Harper and Poole, 2010; Unsworth et al., 2011; Poole et al., 2004).

1.5.4 PI3K regulatory protein

PI3K has a role in sustaining platelet activation and providing stablisation of the thrombus plug. PI3K class I isoforms phosphorylate PIP_2 to produce phosphatidylinositol 3,4,5-triphosphate (PIP_3) which leads to recruitment of proteins with a Pleckstrin Homology (PH) domain to the plasma membrane, where they come in close proximity to the downstream signaling proteins (Bye et al., 2016). Moreover, PI3K has a minor role downstream of GPVI (collagen receptor) on the activity of extracellular signal-regulated kinase (ERK). It has also a role on the colocalization of Btk and its substrate PLCγ2 at the plasma membrane (Bobe

et al., 2001). PI3Kγ is predominantly involved in downstream signaling of P2Y12 (ADP receptor) but has a semi-redundant effect with PI3Kβ which is downstream of GPCR.

1.6 Conditions resulting in platelet hyperactivity

An abnormal increase of platelet activation is seen in a variety of conditions such as cardiovascular disease, diabetes, hyperglycemia and also inflammatory diseases such as atherosclerosis (Lukasik et al., 2010). The development of atherosclerosis begins with formation of fatty plaque. The rate of atherosclerosis varies among individuals and is influenced by a variety of different factors such, both genetic and environmental. Unhealthy diet, smoking, drinking alcohol, diabetes in tandem with low exercise can lead to increased cholesterol in the blood, which in turn contributes to plaque formation. Indeed, obesity plays important role in atherosclerosis progression (Howard et al., 1998; Goff et al., 2014). When a plaque ruptures, it exposes thrombogenic proteins such as collagen and VWF, which leads to activation and aggregation of platelets to the vessel wall and growth of the thrombus (Virmani et al., 1999; Clark et al., 2015). This can lead to development of Acute Coronary Syndromes (ACS), a group of conditions used to describe acute myocardial ischemia which occurs due to formation of a thrombus at a high-risk site leading to an increased risk of MI and stroke. If ACS is not treated quickly, the conditions can lead to death. Indeed, ACS is one of the top five causes of death in the UK (Charles River Associates, 2011). Platelet hyperactivity plays a critical role in the development of ACS. Drugs to reduce platelet activity in such patients are an important pharmacological tool (Laine et al., 2016) thereby reducing the risk of activation by the increasingly thrombogenic stimuli.

1.7 Antiplatelet therapy:

Inhibiting platelet activity and function in ACS patients may help in stopping the reoccurrence of thrombus formation at the site of the plaque. Although initiation of platelet activation occurs by collagen, VWF and thrombin as subendothelial matrix agonists, ADP is critical as a secondary positive feedback, following its release from platelet dense granules (Murugappa and Kunapuli, 2006). Therefore, ADP receptor (specifically P2Y12) inhibitors are the most direct treatment approach.

However, a number of other approaches to inhibiting platelet therapy are under development and are mainly targeted at the α_{IIb}/β_3. The formation of platelet thrombi is determined by the integrin α_{IIb}/β_3-mediated interactions of platelets with fibrinogen and fibrin. The importance of α_{IIb}/β_3 can be seen in patients with Glanzman thrombasthenia, which is caused by mutation of α_{IIb}/β_3 - patients show severely reduced or absent platelet aggregation (Kannan and Saxena, 2009). Table 1.2 provides a brief overview of these approaches. Since P2Y12 inhibition is the focus of the present work, these compounds are not discussed further in this thesis.

Table 1-2 Novel anti-platelet compounds under investigation

Compound	**Drug Type**	**Target**	**Evidence of benefit**
Abciximab	Chimeric human Murin antibody	α_{IIb}/β_3 antagonist	EPIC and EPILOG trials show patients on abciximab, heparin and Aspirin have a 35% reduction in death, MI and ischemic events in comparison with aspirin or heparin only. EPISTENT trial showed the benefit of using abciximab in patient with coronary stenting – reduction in MI and also reduced in bleeding in the abciximab arm.
Eptifibatide	Heptapeptide	Binds reversibly to β_3 on α_{IIb}/β_3	PURSUIT trial demonstrated only a 1.5% absolute reduction in the incidence of the primary end point (death or MI) when compared to placebo. IMAPCT II trial comparing low dose and high dose eptifibatide with placebo, showed no significant difference in rates of death/MI between high dose and placebo, while low dose treatment reduced end-point rates from 11.6% to 9.2%.
Tirofiban	Nonpeptide derivative	α_{IIb}/β_3 inhibitor	RESTORE trial involved patients undergoing PCI and have had MI or unstable angina. Tirofiban was found to protect against early adverse cardiac events related to thrombotic closure, when compared with placebo. At 30 days however, the reduction in adverse cardiac events was no longer statistically significant.

1.7.1 Aspirin

Aspirin or acetylsalicylic acid (ASA), is derived from salicylic acid which is found in the bark of the willow tree. Acetylsalicylic acid has been widely used as an anti-inflammatory, anti-pyretic and anti-thrombotic where it acts to block prostanoid production, a group of eicosanoids which include the prostaglandins, thromboxanes and the prostacyclins (Antman et al., 2005). Prostanoid production depends on the activity of the two isoforms of the cyclooxygenase (COX) enzymes within cells: COX-1, which is present in most cells and its expression is generally constitutive, and COX-2, for which, in platelets, is expressed at low levels but is increased massively upon platelet activation. Upon contact of the platelet with collagen in the sub-endothelial surface, GPVI receptor signalling leads to release of arachidonic acid (AA) by phospholipase A2 (PLA2). This, in turn leads to COX-1 signaling which leads to production of prostaglandin G2 (PGG2), prostaglandin H2 (PGH2) and finally TXA2 (Smith, 1992). TXA2 is released from activated platelets and binds to its TP receptor on the platelet surface for secondary activation. Aspirin irreversibly binds to cyclooxygenase-1, ultimately leading to TXA2 inhibition (Loll et al., 1995; Warner et al., 2010). Ingested aspirin is quickly absorbed from the intestine into blood where it reaches peak levels after one hour (Patrono et al., 2004). Then it is cleared from the blood circulation after it has been metabolised by the liver within 2 hours (Michelson, 2013)

. Due to the anucleate nature of platelets aspirin limits the ability for platelets to generate new COX-1. Therefore, aspirin inhibitory action remains for the life span (7-12 days) of the platelet (Burch et al., 1978). However, normal thrombopoietic turnover in humans means that approx. 10% of the circulating platelets every day have been newly released from the bone marrow, which can be a limiting factor in cardiovascular patients. Drug dosing regimens are critical in ensuring continued effects (Di Minno et al., 1983). Further

limitations of aspirin (particularly at high doses); include side effects such as nausea, gastric ulcer and bleeding (Serebruany et al., 2005), and in rarer cases renal toxicity (The SALT Collaborative (Group, 1991). Lower doses of aspirin can also lead to gastrointestinal bleeding. Despite this, aspirin is used often as a first line antiplatelet treatment for ACS patients, MI and unstable angina.

1.7.2 ADP receptor antagonists

As mentioned previously, ADP binds to two different receptors on the platelet surface, P2Y12 and P2Y1. The initial response to ADP occurs via P2Y1, which induces platelet shape change and Ca^{2+} mobilization. The P2Y12 receptor is the predominant receptor involved in the ADP-stimulated activation of the glycoprotein IIb/IIIa receptor. Activation of the glycoprotein IIb/IIIa receptor results in enhanced platelet degranulation and thromboxane production, and prolonged platelet aggregation (Hechler et al., 2005). ADP also enhances secretion of other material from platelet granules such as P-selectin that plays a role in leukocyte intereaction (Wijeyeratne and Heptinstall, 2011).

The importance of ADP in platelet function via its receptors P2Y1 and P2Y12 highlights a role for these two receptors as targets for antithrombotic drugs. However, the P2Y1 receptor is not platelet specific, expressed in many tissues in the body, which reduces the feasibility of using it as a target for antithrombotic agents (Wijeyeratne and Heptinstall, 2011). Thus, P2Y12 is the main focus of anti-platelet drug discovery.

P2Y12 inhibitors consist of two types of drugs; theinopyridines (ticlopidine, clopidogrel, prasugrel) and the nucleoside-nucleotide agents (cangrelor, ticagrelor, and Elinogrel)

(McFadyen et al., 2018). Theinopyridine drugs are a prodrug which require metabolism by liver cytochrome CYP450 (P450) to be active, and they bind to P2Y12 irreversibly (Kowalczyk et al., 2009; McFadyen et al., 2018). However, the other group of P2Y12 inhibitors bind directly to P2Y12 and do not need to be metabolised by P450) (McFadyen et al., 2018).

Ticlopidine

It is first drug become available to inhibit P2Y12. It is orally administrated and requires activation by cytochrome P450 (Farid et al., 2007). However, this drug proved to have many limitations including hepatic toxicity, thrombotic thrombocytopenic purpura and aplastic anaemia (Steinhubl et al., 1999; Wijeyeratne and Heptinstall, 2011). Moreover, many patients experienced less severe but nevertheless unwated side effects, such as nausea, diarrhoea and vomiting (Michelson, 2013). Therefore, this treatment was phased out and replaced by another thienopyrine family member clopidogrel.

Clopidogrel

Like ticlopidine, clopidogrel binds irreversibly to the P2Y12 receptor to inhibit its effect when stimulated with ADP (Farid et al., 2010). Although the side effects of this drug were shown to be less severe than ticlopidine, symptoms such as gastrointestinal bleeding have been reported. Clopidogrel has been tested for its safety against aspirin for preventing cardiovascular diseases. The CAPRIE (Clopidogrel versus Aspirin in Patients at Risk of Ischemic Event) trial recruited 19000 patients with cardiovascular disease. The results showed that clopidogrel was more effective than aspirin in reducing ischemic stroke, myocardial infraction, vascular death and bleeding (Lancet, 1996). Since then clinical trials

have been conducted involving dual antiplatelet therapy of aspirin in combination with clopidogrel. The CHARIMA trial compared use of this duel therapy against aspririn alone and although it showed benefit in some patients, overall it was reported that aspirin and clopidogrel was not significantly more effective than aspirin alone in reducing the rate of myocardial infarction, stroke, or death from cardiovascular causes (Bhatt et al., 2006). In contrast, the CURE trial demonstrated beneficial effects of duel therapy in patients with ACS without ST-segment elevation. However, the risk of major bleeding was increased among patients treated with clopidogrel (Mehta et al., 2001).

With regard to clopidogrel dosing, the CURRENT-OASIS 7 and CREDO trials demonstrated that high doses of clopidogrel have beneficial effects in preventing ischemic events, when compared to low doses. However, high doses are associated with increased bleeding tendency, while low doses are associated with continued platelet hyperactivity (Mehta, 2010). Despite its relative success, it is reported that 4-30% of patients show no or poor response to clopidogrel (Clark et al., 2015).

There are several factors that can influence the effectiveness of clopidogrel. The major factor is CYP2C19*2 loss of function allele, resulting in impaired metabolism of the compound (Johnson et al., 2012; Clark et al., 2015). P2Y12 receptor gene polymorphisms have also been noted (Bouman et al., 2010). Patients with specific comorbidities such as diabetes mellitus, intestinal conditions and impaired renal function will also show reduced response to the drug (Gremmel et al., 2011; Massarelli et al., 2011; Damman et al., 2012).

Prasugrel

The third generation of oral thienopyridine, prasurgrel irreversibly binds to P2Y12. Compared to clopidogrel, prasugrel has a rapid onset of action, blocking the P2Y12 receptor, and reaching maximum concentration in the blood after approximately 30 minutes of oral administration. It is also metabolised by cytochrome P450 in the liver (Sugidachi et al., 2007; Michelson et al., 2009). Results from the TRITON-TIMI38 trial demonstrated that prasugrel ($P<0.001$) is more effective than clopidogrel in reducing ischemic events and myocardial infarction. However, high risk of bleeding was noted in elderly patients (>75 years) with history of stroke (Wiviott et al., 2007). Results from the JUMBO-TIMI study found significantly more bleeding events in patients taking Prasugrel compared to patients taking Clopidogrel (Damman *et al.,* 2012; Sarafoff *et al*., 2012). Interestingly however, in a randomized trial Kim et al. (2016) found that prasugrel overcame the poor outcome for the CYP2C19*2 loss-of-function allele seen when treated with Clopidogrel (Kim et al., 2016).

Despite the development of prasurgrel, clopigorel and aspirin still remain the mainstay of treatment in the UK for patients at risk of thrombotic events.

Cangrelor

Cangrelor was the first of the direct reversible P2Y12 inhibitors, administrated intravenously (Storey et al., 2001). Cangrelor is from the ATP analogue family and has a very short half-life of 3-6 minutes. As a result, normal platelet function is restored after 1 hour of discontinuing the treatment (Storey et al., 2001; Angiolillo et al., 2012). This is extremely useful in cases where bleeding begins to occur, and the clinician needs to rapidly

reverse the effects. However, a phase III trial showed that cangrelor does not provide superiority in terms of primary end-point, MI or ischemic driven revascularization compared to clpidogrel (Harrington et al., 2009; Bhatt, 2009). In contrast, the CHAMPION PHONIX trial reported benefits of cangrelor in reducing ischemic events without major bleeding (D. L. Bhatt et al., 2013).

Ticagrelor

Ticagrelor (AZD6140) was the first orally administered reversible P2Y12 inhibitor to be developed (Kowalczyk et al., 2009). The PLATO trial reported a decrease in thrombotic events for patients treated with ticagrelor compared with clopidogrel (Wallentin et al., 2009). However, ticagrelor has been associated with a higher bleeding risk than clopidogrel, but reduced bleeding risk compared with cangrelor and prasugrel (Damman et al., 2012).

Elinogrel

Similarly to ticagrelor, elinogrel is a direct reversible P2Y12 inhibitor with rapid onset of action (Cattaneo and Podda, 2010). Although well tolerated in trials involving healthy participants, and demonstration of strong anti-platelet activity, the ERASE-MI randomized trial evaluating safety and tolerability of intravenous elinogrel (10, 20, 40, and 60 mg) before PCI in patients with STEMI, showed no differences in serious adverse events, laboratory values, corrected thrombolysis in Myocardial Infarction frame count, or ST resolution when compared with those parameters observed in placebo-treated patients. Furthermore, the INNOVATE-PCI trial, comparing eliogrel and clopidogrel treatments in patients undergoing non-urgent (including elective) PCI found no difference in efficacy or bleeding tendency at the 24-hour and 120-day time points. However, eliongrel was shown

to achieve platelet inhibiton more quickly than clopidogrel. Despite this, development of the drug was terminated in 2012 (Müller et al., 2012). An overview of the P2Y12 inhibitors can be seen in table 1.3.

Table 1-3 Current P2Y12 inhibitors and their properties.

P2Y12 Inhibitor	Type	Administration	prodrug	Type of binding	Dosage	Initiation time of drug	Clearance time of the drug
Ticlopidine	Thienopyridine	Oral	Yes	Irreversible	2x 250 mg	3-5 days	4-5 days
Clopidogrel	Thienopyridine	Oral	Yes	Irreversible	75mg*	3-4 hours	7 days
Parasugrel	Thienopyridine	Oral	Yes	Irreversible	10mg	1-2 hours	7 days
Ticagrelor	CPTP	Oral	No	Reversible	2x90mg*	1-2 hours	5 days
Cangrelor	ATP analogue	Intravenous	No	Reversible	30µg/kg bolous and 4µg/kg/min infusion	15-30 min	1 hour
Elinogrel	Sulphonyle derivative	Oral and intravenous	No	Reversible	60mg orally Oral: 50–150mg Intravenous: 80 or 120 mg bolus twice daily	Not characterized yet.	12 h

*Initial dose: clopidogrel 300-600 mg, parasugrel 60 mg,and ticagrelor 180 mg

1.8 Multifaceted roles of platelets

1.8.1 The role of platelets in inflammation and immunity

The role of platelets in haemostasis has long been understood. However, recently, there is growing appreciation that platelets also function in immune and inflammatory processes (Morrell et al., 2014). Platelets are able to recruit leukocytes to the site of vascular damage and inflammation by releasing pro-inflammatory mediators such as PDGF, PF4 and transforming growth factor –β (TGF-β) (Freedman, 2003; Smyth et al., 2009). Furthermore, platelets also have the ability to bind to leukocyte receptor P-selectin glycoprotein ligand-1 (PSGL-1) via interaction with CD62P (P-Selectin) on the platelet surface, forming platelet-leukocyte aggregates. *In-vivo* work has demonstrated the importance of these platelet–leukocyte aggregates in vascular disease, as infusion of recombinant human PSGL-1 in an animal model of vascular injury reduced myocardial reperfusion injury and preserved vascular endothelial function (Freedman, 2003). CD62P also acts as an initiator for the complement system, thereby playing a pivotal role in orchestrating phagocytosis of invading pathogens (Smyth et al., 2009).

ADP released by dense granules upon platelet activation, binds to the P2Y12 receptor on dendritic cells (DCP2Y12), leading to increased antigen endocytosis (Morrell et al., 2014). Collectively, the literature shows that in addition to the major roles in haemostasis, many granule-derived molecules also have secondary functions in immunological processes. A summary of granules' exocytosis and their role in inflammatory and immune system in table 1.4.

Table 1-4 List of important platelet-derived inflammatory and immune mediators (Morrell et al., 2014).

Storage vesicle	Molecule	Immune/Inflammatory role
α-granule	PF4	Chemokine: monocyte, neutrophil, and T-cell recruitment, macrophage phagocytic activity.
	P-selectin	Selecting: leukocyte adhesion, complement activation.
	CD40L	TNF superfamily: antigen-presenting cell activation.
	TGF-β	Cytokine: cell proliferation, T-cell differentiation, B-cell and macrophage phenotype regulation.
	PDGF	Growth factor: cell growth and differentiation, monocyte/macrophage differentiation.
	VWF	Platelet adhesion, PMN extravasation.
	CD63	Tetraspanin: transmembrane adaptor protein, leukocyte recruitment.
	SDF-1	Chemokine: T-cell, monocyte, and PMN chemotaxis.
	VEGF	Growth factor: angiogenesis, adhesion molecule expression.
	Thrombospondins	Apoptosis, endothelial cell inflammation, macrophage-platelet aggregates.
	MIP-1α	Cytokine: neutrophil and eosinophil activation, B-cell immunoglobulin production.
	MMP-2, MMP-9	Protease: extracellular matrix breakdown, platelet-leukocyte aggregate formation
	Cyclophilin A	Vascular smooth muscle cell growth factor.
Dense Granule	Serotonin	DC and T-cell functions.
	Glutamate	T-cell trafficking
	Polyphosphates	Inflammatory response amplification
	ADP	Platelet, leukocyte, endothelial cell activation.
	Histamine	Increased vessel reactivity and degranulation.

Produced or constitutively expressed	IL-1β	Cytokine: acute phase response, leukocyte and endothelial activation.
	Thromboxane	Eicosanoid: T-cell differentiation, monocyte activation.
	Nitric oxide	Reactive oxygen species: anti-inflammatory and antithrombotic.
	GPIbα	Adhesion molecule: binds Mac-1 on leukocytes.

1.8.2 The role of platelets in malignancy

Platelets contain more than 30 angiogenesis proteins that may contribute to the promotion of new blood vessel growth, particularly in the developing tumour. These include Vascular Endothelial Growth Factor (VEGF), Basic Fibroblast Growth Factor (bFGF), PDGF, Epidermal Growth Factor (EGF) and matrix metalloproteinase (MMPs) (Italiano et al., 2008) the most important of these in angiogenesis is VEGF. However, platelets also contain anti-angiogenic factors such as PF4, endostatin and Thrombospodin-1 (TSP-1) (Bambace and Holmes, 2011).

Cancer cells themselves may express or secrete platelet agonists such as Tissue Factor (TF), ADP and thrombin. This promotes adhesion of platelets to the tumour cells and consequent activation of the platelets. This activation leads to the release of pro and anti-angiogenic proteins from the platelets. The mechanism of each angiogenic factor is illustrated below (Daly et al., 2003).

It has been reported that there is a direct relationship between TF expression and VEGF concentrations, where high concentrations of VEGF in cancer patients correlates with

increased expression of TF by endothelial cells in the tumor vasculature (Bambace and Holmes, 2011). This expression may explain the increased incidence of thrombosis in cancer patients. Furthermore, Thrombin concentrations have been reported to increase dramatically in cancer patients compared to healthy subjects and this also promotes activation of platelets and release of their angiogenic proteins (Bambace and Holmes, 2011).

If platelets contain both pro-angiogenic and anti-angiogenic factors, stimulating release of both of these equally should neither promote nor inhibit angiogenesis. However evidence suggests that there is selective release of such factors; Activation of platelets via ADP-P2Y12 receptor interactions results in the release of VEGF but not endostatin, leading to an overall pro-angiogenic effect in this setting (Sabrkhany et al., 2011). A review by Almog et al. (2010) highlights the importance of balancing pro- and anti-angiogenic factor release by platelets on tumour progression (Fig. 1.4). In a healthy mouse, these factors are in fine balance, while in mice with dormant or inactive cancers there is an imbalance, in favour of the anti-angiogenesis proteins. In contrast, mice with active tumours express higher levels of the pro-angiogenic factors (Almog and Klement, 2010).

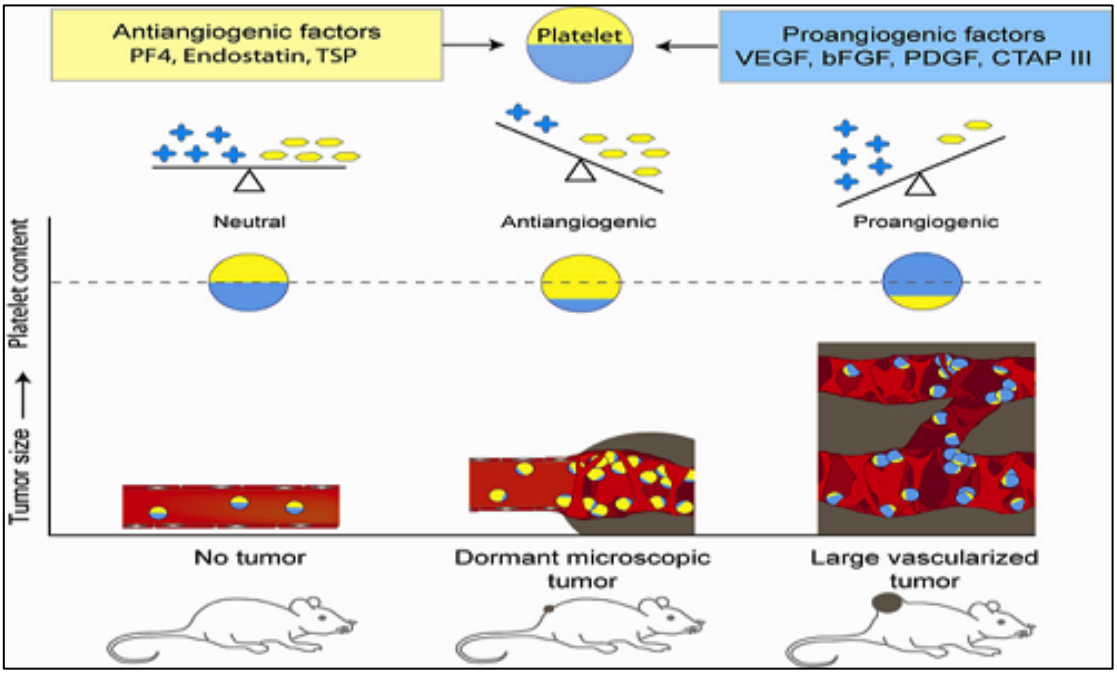


Figure 1.4 Importance of balancing pro- and anti-angiogenic factors. In the healthy mouse model (left), there is balance between Anti-angiogenic (Yellow circle symbol) and Pro-angiogenic (blue plus symbol) factors, while in the mouse with dormant or inactive cancer (centre), there is an imbalanced level of these proteins which is in favour of anti-angiogenic proteins. In the active cancer model (right), the

In addition to releasing factors that may promote cancer growth, platelets can also uptake or sequester some tumor-derived proteins that appear essential for tumor metastasis (Bambace and Holmes, 2011). A study by Klement (2004) revealed that the concentrations of angiogenic proteins inside platelets from animals bearing malignant cancer were significantly higher than in platelets from healthy animals. Furthermore, when VEGF was injected into these mice subcutaneously, the level of VEGF in platelets increased without significantly changing the overall plasma concentration, providing evidence that platelets selectively uptake certain proteins from their microenvironment (G. L. Klement et al., 2009). This is selective as other proteins such as albumin, that are also at high concentration in the plasma are not taken up by platelets (G. L. Klement et al., 2009).

Platelets isolated from glioma and prostate cancer patients have been found to contain tumor associated RNA, EGFRVII and PCA3. It is proposed that these could be considered as biomarkers and analysis of platelet contents in such patients could be a useful, non-invasive test for the diseases (Nilsson et al., 2011). Specific gene expression signature in platelets from cancer patients also suggests that analysis of platelets may be useful in the diagnosis of cancer and the monitoring of disease progression (Nilsson et al., 2011). Literature evidencing the uptake of tumour-derived proteins is summarized in Table 1.5.

Table 1-5 Summary of literature evidencing the uptake of tumour-derived proteins by platelets in different cancer types

Author	**Cancer type**	**Sequestered tumour-derived factors by platelets**
Klement et al., 2009	Malignant animal – breast cancer	VEGF
Nilsson et al., 2011	Glioma and prostate cancer	Tumour associated RNA (EGFRVII and PCA3)
(Kerr et al., 2010)	Human prostate cancer cell	TGF-β_1, VEGF, MCP-1, MMP-2, RANK, RANKL and TIMP-1.
(Fu et al., 2015)	Non-small cell lung cancer (NSCLC)	VEGF
(Benoy et al., 2002)	Breast cancer	VEGF
(G. Klement et al., 2004)	Human tumour injected in mice	VEGF, bFGF, PDGF, BDNF, endostatin
(Peterson et al., 2012)	Colorectal cancer	VEGF, PDGF and PF4
(Cervi et al., 2008)	Liposarcoma	PF4

The uptake of tumour-derived proteins and cytokines by platelets is thought to protect these molecules from degradation in the general circulation (Kerr et al., 2010). Tumor-derived VEGF and TGF-β_1 which aid tumor progression and metastasis, are preferentially taken up and stored in platelets, where their levels are 1.4 and 1.96 fold higher respectively than in plasma (Kerr et al., 2010). As a result of this sequestration of tumor proteins, it is suggested that platelets can play important roles in metastatic processes by transporting these factors to distant sites within the body (Kerr et al., 2010). Furthermore, platelets also have a protective effect on tumor cells, binding to them via platelet activating factor and shielding them from immune detection by Natural Killer (NK) cells. This also enhances extravasation and metastasis (Bambace and Holmes, 2011). The uptake of relatively high concentrations of tumour-derived factors could affect the haemostatic function of platelets, but to date, solid conclusions have not been made.

1.8.2.1 Evidence of Haemostatic abnormalities in cancer patients.

Armand Trousseau first described the link between cancer and haemostatic abnormalities in 1865. Thereafter, unexpected and unexplained thrombotic problems in carcinoma patients was termed Trousseau syndrome (Varki, 2007). The underlying mechanisms are still not understood and may be multifaceted. Tissue Factor (TF) expression and up-regulation of the thrombin receptor (PAR-1) by some cancer cells are two proposed explanations for this link. TF and Thrombin receptor expression cause thrombosis either locally at the site of the tumor, or at distant sites in the body (Varki, 2007). However, some patients with low expression of TF and thrombin receptor may still present with thrombotic complications, suggesting alternative underlying

mechanisms (Falanga et al., 2013). A large number of studies report on the percentage of thrombosis or active bleeding in cancer patients (Table 6.2). Surprisingly, incidence of venous thromboembolism (VTE) in cancer patients is reported to be seven fold higher than in a healthy population (Falanga et al., 2013). On the other hand, active bleeding is also seen in cancer patients and is an important cause of death in 10% of solid tumor patients, a figure which is suggested to be even higher in patients with haematological malignancies (Falanga et al., 2013).

Klastersky et al. (1971) reported severe haemorrhage in patients with acute leukemia where 30% of patients died as a result. Although thrombocytopenia is often a symptom of this disease, it was proposed that this decrease in platelet count was not the only factor involved in the fatal bleeding, suggesting that thrombocytopathy in the presence of adequate platelet counts may also be important (Klasters.J et al., 1972).

1.8.2.2 The link between tumour-derived proteins and platelet function

Tumour-derived proteins may interfere with platelet function and may lead to thrombosis (hyperactivity) or risk of bleeding (hypoactivity). A large body of evidence exists documenting changes in serum concentrations of cytokines and growth factors in cancer patients, compared to control patients (examples in table 1.6), and so it is not unreasonable to propose that this may be the cause of the observed haemostatic disturbances. Interestingly, the collective data highlight several cytokines that are at higher concentrations in the serum of patients with different types of cancer, notably IL-6, TNF-a and VEGF. Furthermore, it is suggested that tumor-derived IL-6 has a role in the regulation of VEGF inside the platelets of breast cancer patients, illustrating the link between IL-6 in serum and VEGF in platelets (Benoy et al., 2002).

Table 1-6 Examples of elevated cytokines in peripheral blood of some cancer patients.

Author	Cancer type	Cytokines in peripheral blood
(Kerr et al., 2010)	Human prostate cancer cell	G-CSF, HGF, IL-1β, MMP-9 and OPG.
(Fu et al., 2015)	Non-small cell lung cancer (NSCLC)	TGF-β_1
(Benoy et al., 2002)	Breast cancer	VEGF, IL-6

Furthermore, Rubenstein et al. (2002) have shown that overexpression of VEGF in an animal model with glioblastoma induces vascular destabilization which lead to intracerebral haemorrhage and spontaneous tumor haemorrhage (Rubenstein et al., 2002). Moreover, Peterson et al. (2012) revealed that platelets from colorectal cancer patients have significant increases in VEGF. PDGF, PF4 (Peterson et al., 2012).

1.8.3 The role of platelets in cancer

Platelets play vital role in haemostasis and thrombosis and recently they have also been involved in inflammatory and angiogenesis process (Morrell et al., 2014; Sabrkhany et al., 2011; Ronnlund et al., 2012; Almog and Klement, 2010). Moreover, it has been found that platelets consider as key mediator of tumour metastasis (Almog and Klement, 2010; (Sabrkhany et al., 2011) . Surprisingly, patients with cancer wether solid tumour or haematological malignancy have abnormal haemostatics parameters, which may lead to thrombosis or active bleeding (Ronnlund et al., 2012). This indicates that cancer cell or neo-angiogenesis process may affect on platelets function in these patients.

There are large body of evidence demonstrate that platelets sequester many microenviromental factors which could be the reason for abnormal function of platelets in the case of cancer patients where cancer cell secrete abnormal level of proteins in the circulation (Peterson et al., 2012; Nilsson et al., 2011; G. L. Klement et al., 2009; G. Klement et al., 2004). Some of these factors that are sequestered in platelets have been found to be cancer specific which may give advantage to platelet to be test as early tumour detection in cancer patients.

Moreover, other studies have reported the percentage of thrombosis and bleeding in cancer patient are increase (Table 6.1). Surprisingly, it has been reported that VET incidence in cancer patients seven times higher than healthy people (Falanga et al., 2013). On the other side, active bleeding has also been seen in cancer patients which

consider as an important cause of death in 10% of solid tumour patients. As results of that haematological malignancy could be higher even than the solid tumour.

1.9 Role of endothelial cells in haemostasis

Clot formation results from the interplay between platelets, coagulation factors and also endothelial cells on the vascular wall (Wu and Thiagarajan, 1996; Verhamme and Hoylaerts, 2006). The main function of the endothelial cells in haemostasis is to maintain blood fluidity and limit thrombus plug formation to a very localised area. Endothelial cells release anti-platelet and anti-coagulant factors to supress platelet activation and fibrin formation. Therefore, endothelial dysfunction not only precedes atherogenesis but may also predispose to arterial thrombosis.

Under normal conditions, endothelial cells release Nitric Oxide (NO), prostacycline (PGI2) (Verhamme and Hoylaerts, 2006) and prostagladine E2 (PGE2). These molecules work to inhibit platelet activation (and reverse platelet aggregation) by increasing cyclic AMP (Cattaneo and Lecchi, 2007). Additionally, endothelial cells present/release a whole host of other factors which act to inhibit platelets, fibrin formation and coagulation factor activation. There are summarised in table 1.7.

During endothelial injury or dysfunction, expression/secretion of these anti-thrombotic properties is reduced/lost, and the cells express pro-thrombotic molecules such as VWF and collagen from the sub-endothelial matrix. This leads to adherence of platelets to VWF and collagen where they become active and release secondary mediators such as ADP and TXA2 (Wu and Thiagarajan, 1996) thereby amplifying the platelet response.

Therefore, inherent platelet hyperactivity is not the only cause of inappropriate platelet aggregation and endothelial dysfunction can lead to the same effect. The role of the endothelium must be considered when investigating disorders of inappropriate thrombus formation.

Table 1-7 Summary of Endothelial molecules function in haemostasis adapted from (Wu and Thiagarajan, 1996).

Endothelial mediators	**Function**
Anticoagulant molecules	Prevent thrombosis
PGI2	Inhibit platelet function
NO	Inhibit platelet function
tPA	Enhance fibrinolysis system
TFPI	Inhibit coagulation factor Xa and VIIa/TF complex.
Thrombomoduline	Modify thrombin substrate to induce protein C activation
Protein S	Enhance protein C to inactivate factor Va and VIIIa.
Heparan Sulfate proteoglycan	Cofactor for antithrombin III
Ecto-ADPase	Inactivate ADP and suppress platelet aggregation
Procoagulant molecules	Form thrombosis at injured tissue to stop bleeding or may cause thrombotic complication at abnormal plaque formation
VWF	Induce platelet adhesion and activate factor VIII.
PAI-1	Inhibit tPA
TF	Cofactor for factor VIIa
Factor Va	Induce activation of factor Xa

1.10 Platelet function tests

Platelet function tests are very important in the investigation of haemostatic disorders and the effects of antithrombotic therapy. Recently, it has become common to use platelet function testing for monitoring antiplatelet therapies in cardiovascular disease patients, allowing determination of the optimal dose of antiplatelet therapy (Michelson et al., 2006).

1.10.1 Light transmission aggregometry (LTA)

LTA is the "gold standard" test for platelet function. The platelet aggregometer consists of a cuvette for the sample and source of light and a detector. The principle depends on turbidity of a sample of platelet-rich plasma (PRP) in the cuvette. When the platelets are in single form and not-aggregated, the sample of light passing through the cuvette is absorbed by the sample. Once an agonist is added, the platelet begins to aggregate the sample becomes translucent and more light passes through the sample. This is seen as a change in absorbance. A sample of platelet-poor plasma (PPP) is used as a comparator for 100% aggregation (Born, 1962; O'brien, 1962; Natalia Dovlatova et al., 2015; McFadyen et al., 2018). The change in absorbance is measured over 5 minutes, and so the method can very quickly give an indication of platelet function in a patient.

1.10.2 Impedance aggregometry

Impedance aggreometry (or Whole Blood Aggregometry, WBA) is based on the principle of electrical impedance by platelets aggregating and binding to an electrode. Two

electrodes are inserted into an anticoagulated whole blood sample and an alternative current is pass between them (May et al., 2004). When platelets are activated by an agonist, they accumulate around the electrode and increase the impedance, which is measure in Ohm (Ω). This increase in impedance is directly proportion to the degree of platelet aggregation (May et al., 2004).

One of the advantages of this method is it does not need any sample preparation as it uses whole blood. Further, the conditions are said to be more reflective of the in-vivo environment, and so any effect of an anti-platelet agent by impedence aggregometry can be assumed to be the same in-vivo. However, in contrast, using a whole blood sample may produce in-vitro artefacts which interfere with the result and hence there is an argument that LTA is the superior method (von Beckerath, 2008).

1.10.3 Platelet function analyzer-100 (PFA-100)

PFA attempts to mimic the in-*vivo* platelet function at the site of injury. The machine consists of a disposal cartridge that has a reservoir for the sample, capillary of coated membrane with collagen/ADP or epinephrine with 150μm aperture. The principle of the machine is based on time occlusion of the aperture by a platelet plug formation (May et al., 2004). This is rapid and simple to perform with less technical skill than LTA or WBA (May et al., 2004).However, this test needs quality control to give optimal results compared with other laboratory and is relatively expensive in comparison with LTA and WBA.

1.10.4 Flow cytometery for platelet activation

Flow cytometry allows for the analysis of platelet surface activation markers. Two of the most commonly analysed markers are CD62P (P-Selectin) which is released from α-granules following platelet activation, and activated $\alpha_{IIb}\beta_3$ which allows for fibrinogen binding. The method provides rapid analysis in PRP and also whole blood samples. In the case of whole blood sample, platelets are often identified in the sample by staining with a platelet-specific CD41, in combination with antibodies targeting the activation markers. The blood sample is pre-incubated with a fluorochrome-conjugated antibody which binds to the target antigen on the platelet surface. If bound, the fluorophore will excite at the specified wavelength and is detected by photomultiplier detector and converted into electronic signal that proportion to the number of antibodies (Ab) on platelet surface (May et al., 2004). This method can be used to predict the risk of recurrent thrombotic event in ACS patients who are under anti-platelet drug such as clopidogrel (Dovlatova et al., 2015).

Flow cytometry can also be used to assess the formation of platelet-leukocyte aggregates, which rapidly form following platelet activation. Expression of CD62P on platelet surface leads to initiate the link with its precursor P-selectin glycoprotein-1 (PSG-1) on leukocytes. This leads to platelet leukocyte interaction, which indicate a better result of platelet activation through P-selectin. Duel labelling with a leukocyte marker (CD45) and platelet marker (CD42b) can be used to identify double-positive events within the sample, and thus platelet-leukocyte aggregates (Gibbins et al., 2004).

1.10.5 Ca^{2+} flux assay

Intracellular Ca^{2+} is a key indicator of platelet activation. Binding of a platelet agonist with its receptor leads to PLC signalling, IP_3 generation and subsequent release of Ca^{2+} from the granules into the cytosol, and also entry of extracellular Ca^{2+} via ion channels in plasma membrane. Elevation in the level of intracellular Ca^{2+} plays an important role in platelet shape change, aggregation and degranulation (Gibbins et al., 2004; Assinger et al., 2015).

Ca^{2+} flux in platelets (and other cells types) is a rapid process occurring within microseconds after addition of the agonist. This poses a challenge for most assays used to investigate changes in intracellular Ca^{2+}. Most studies utilise confocal microscopy, plate-based assays, spectrofluorometry, and standard flow cytometry for such measurements, although there are issues with the number of cells that can be analysed or gaps in recording due to the addition of stimulants, with significant loss of detail of a rapid Ca(2+) response (Vines et al., 2010). Continuous flow cytometry, on new generation flow cytometers allows for constant analysis of a sample before, during and after addition of a stimulant. This has revolutionalised the study of Ca^{2+} signalling as spikes in intracellular Ca^{2+} are seen in real-time. There are several indicator dyes that can be used to bind to cytosolic Ca^{2+} including Fura-2, Indo-1, Fluo-3 and Fluo-4. These dyes passively cross the plasma membrane and bind to cytosolic Ca^{2+}, reporting any spike in intracellular concentration has an increase in fluorescence (Morgan, 1993; Gibbins et al., 2004; Assinger et al., 2015).

1.11 Thesis Aims:

Platelets have both haemostatic and inflammatory roles and platelet dysfunction leads to a wide variety of conditions. For haemostatic disorders such as ACS, which may result in MI or stroke, modulation of platelet activity is vital. However, current anti-platelet therapies have their limitations; those used extensively such as aspirin and clopidogrel do not show effectiveness in all patients, and hyperactivity can continue in patients taking these drugs. In contrast newer agents such as ticagrelor show superiority in terms of platelet inhibition but are associated with increased bleeding tendency. There is therefore a need for continued development and refinement of anti-platelet therapies.

This study aimed to investigate the use of novel thienopyridine derivatives as anti-platelet agents. The study achieved this aim via completion of the following objectives:

Objectives:

1. Analysis of the effect of six novel thienopyridine derivatives on the platelet activation and function, and comparison of these effects with those observed following use of conventional anti-platelet therapies
2. Analysis of the effect of the novel thienopyridine derivatives on the platelet signalling pathways, and comparison of these effects with those observed following use of conventional anti-platelet therapies.
3. Analysis of the effect of these novel thienopyridine derivatives on endothelial function, in terms of their ability to supress agonist-induced adhesion molecule expression

4. The ability of microenvironmental factors (using a co-culture model) to affect platelet function, with the aim of providing a basis for future investigation of the effects of anti-platelet therapies on the immunological function of platelets.

Chapter 2: Materials and Methods

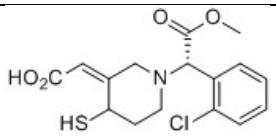
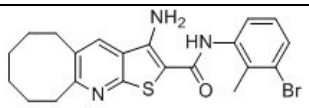
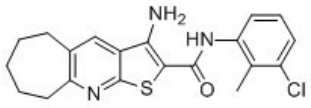
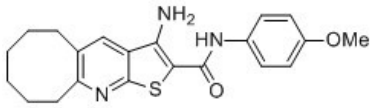
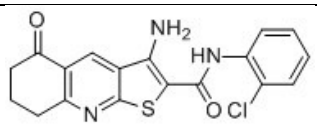
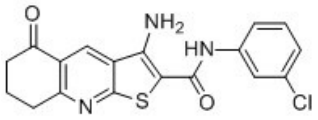
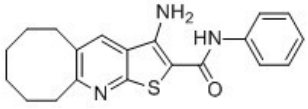
Chapter 2: Materials and Methods

2.1 Thienopyridine derivative synthesis

Thienopyridines were synthesised by our collaborators at the School of Chemical Sciences, University of Auckland, New Zeland, and as previously described (Leung *et al.*, 2014; Hung *et al.,* 2014; Arabshahi *et al.*, 2015; Leung *et al.*, 2016). Briefly, the appropriately 5,6-disubstituted-2-thioxo-1,2-dihydropyridine-3-carbonitriles were condensed with substituted 2-bromo-*N*-phenylacetamides to give the desired thieno[2,3-*b*]pyridines. All compounds were obtained as pure solids after recrystallisation from an alcoholic solvent and their structures were confirmed using NMR and mass spectrometry.

The molecular structures of the six derivatives are demonstrated (Table 2.1).

Table 2-1 Chemical structure of thienopyridine derivatives used in this study

Name	Molecular Weight	Chemical Structure
Clopidogrel active metabolite	355.5	
DJ0206	444.39	
DJ0081	385.91	
DJ0021	381.49	
DJ0171	371.84	
DJ0097	371.84	
DJ0199	351.47	

2.2 Ethics

The Manchester Metropolitan University ethics board granted approval for the study. Healthy volunteers were recruited for blood collection and gave written informed consent before donating a blood sample. Participants who had taken anti-platelet medication, anti-inflammatory medications, herbal medicines that may interfere with platelet function (Ginko Biolba, St John's Wort) or Selective Serotonin Reuptake Inhibitors in the past fortnight were excluded from the study. The study involved a total

of sixty-two participants (n=40 male, n=22 female, age range 20-38). Samples from some participants (chosen at random) were used for analysis of multiple markers of platelet activation and function.

2.3 Sample Collection and Platelet-rich Plasma (PRP) purification

Blood samples were collected from the median cubital vein of healthy volunteers by using sodium citrate vacutainer tubes. The vacutainers were centrifuged at 180 *g* for 15 minutes at 20 °C. Then Platelet Rich Plasma (PRP) was aspirated carefully from the top layer and transferred to a fresh tube. The centrifugation was stopped with zero break to avoid remixing the PRP with the red blood cell layer.

PRP was then diluted at a 1:1 ratio with Tyrode's buffer (NaCl (134 mM), KCl (2.9 mM), Na_2HPO_4 (0.34 mM), $NaHCO_3$ (12 mM), $MgCl_2$ (1 mM), HEPES (20 mM), Glucose (5 mM), adjusted to pH 7.4). Platelet Poor Plasma (PPP) was also prepared for the Light Transmission Aggregometry (LTA) experiment – A 500 µl volume of diluted PRP was centrifuged at 5000 *g* for 5 minutes at 20°C to aspirate PPP from the platelet pellet.

2.4 Washed platelet preparation

Blood was collected in Acid Citrate Dextrose (ACD) vacutainer tubes. PRP was prepared as in section 2.3, and apyrase 0.2u/ml (Sigma-Aldrich, UK) was added to the PRP. The sample was centrifuged again at 2200g for 10 min at 20°C to obtain the platelet pellet and the supernatant was discarded. The pellet was resuspended in Tyrode's buffer containing 0.35% Bovine Serum Albumin (BSA) (Sigma-Aldrich, UK). The platelet count was then adjusted to $8*10^8$/ml in Tyrode's buffer.

2.5 Drug treatments

Unless otherwise specified, for experiments involving PRP, a 400µl volume of diluted PRP was treated with 50µl thienopyridine (final concentration 10µM) for 30mins at 37°C. In experiments involving whole blood, 500µl whole blood was treated with 500µl thienopyridine (final concentration 10 µM) or vehicle control for 30mins. In ASA synergy experiments, 400µl samples of diluted PRP were treated with 25µl thienopyridine and 25µl ASA (final concentration 30M). Following treatment diluted PRP or whole blood samples were analysed using light transmission aggregometry or impedance aggregometry respectively.

2.6 Platelet Activation Studies by Flow cytometry

Flow cytometry was used to assess platelet activity by labelling platelets with fluorochrome-conjugated anti-CD62P (BD Bioscience, UK, Cat: 555524) and PAC1 (BD Bioscience, UK, Cat: 091515). CD62P expression gives an indication of alpha granule release, while PAC-1 binding reflects expression on the activated fibrinogen receptor (GP IIb/IIIa). A 90µl volume of control or treated PRP was stimulated with 10µl of 10µM ADP (Sigma-Aldrich, UK) before addition of 10µl PE-conjugated CD62P (BD Biosciences, UK) and 10µl FITC-conjugated PAC1 (BD Biosciences, UK) and incubation for 10 minutes at 20^{o}C. A 100µl volume of 4% paraformaldehyde (PFA) (Sigma, UK) was then added to arrest any further reaction, holding the platelets in an activated state. A 300µl volume of DPBS (Lonza, UK) was added and samples were analysed on the FACS Verse flow cytometer (Becton Dickinson). Each sample was run in duplicate. Platelets were gated using FSC/SSC for size/granularity, and FITC and PE Mean Fluorescence Intensity (MFI) were recorded.

Platelet leukocyte aggregates (PLAs) were used to further assess platelet activation. A 5µl volume of control or thienopyridine-treated whole blood was diluted with 55µl DPBS and then stimulated with a 10µl volume of 10µM ADP. A 10µl volume of each of PE-conjugated CD45 (BD Biosciences, UK) and FITC-conjugated CD42b (BD Biosciences, UK) were added to the mixture and incubated for 20 minutes at 20^{o}C. Samples were then diluted with 1ml DPBS and analysed immediately on the FACS Verse flow cytometer. Each sample was run in duplicate.

2.7 Light transmission aggregometry (LTA)

The drug-treated PRP samples (450 µL volume) were placed in glass cuvettes with a stir bar and placed in the Chronolog 700 aggregometer (Chronolog, USA) at 37^{o}C. After 1 minute of baseline reading, 50µl of ADP (final concentration 10 µM) (Labmedics, UK) or collagen (final concentration 1 µg/ml) (Chronolog-Corp, USA) was added and the aggregation (MaxA) was recorded for a further 4 minutes. *A* 500µl volume of PPP was placed in the analyser for calibration to 100% light transmission. Each sample was run in triplicate.

2.8 Ca^{2+} Assay

For the Ca^{2+} assay, washed platelets were prepared. Citrated whole blood was centrifuged at 200g for 20 mins. The PRP was carefully aspirated from the red cell layer and transferred into a fresh tube. A 2U/ml concentration of apyrase was added to the sample. The sample was further centrifuged at 1800 g for 10 minutes and the plasma

was removed from the platelet pellet. The pellet was resuspended in 5ml Tyrodes-Hepes buffer (NaCl 134 mM, KCl 2.9 mM, Na_2HPO_4 0.34 mM, $NaHCO_3$ 12 mM, $MgCl_2$ 1 mM, HEPES 20 mM, Glucose 5 mM, pH 7.4). A 1 µl volume of 4µM Fluo4-AM (Invitrogen, UK) and 0.2% pluronic was added to 1000 µl of the sample which was incubated for 45 min @37°C in the dark. The sample was centrifuged at 1800xg, 10min to remove excess dye. The platelets were then diluted in Tyrode's buffer to the desired density (2-4 x 10^8cells/ml). Finally 0.162u/ml apyrase was added again to reduce de-sensitization of the ADP receptors prior to analysis.

The Fluo4-AM loaded washed platelet samples were then treated with thienopyridine for 30 mins and were then analysed on a Accuri C6 Flow Cytometer, where ADP was used as the agonist during sample acquisition.

2.9 Cell culture

The HUVEC cell line (ATCC, Middlesex, UK) was used in this study to test the effect of thienopyridine compounds on endothelial cells. As these cells are adherent cells they were cultured in 1% Gelatin coated flasks (Thermo Scientific, UK) using M199 culture medium (Lonza, UK) containing 10% Fetal Bovine serum (FBS) (Gibco, Switzerland) and maintained at 37^0C in a humidified environment of 5% CO_2 in air. The passagings of these cells were performed by routine trypsinisation (Lonza, UK) and replacement of fresh M199 medium. Cell viability was assessed using the Trypan blue exclusion method on a haemocytometer, and experiments were only performed when cell viability was >95%.

The Hodgkin lymphoma cell line L1236 (ATCC, Middlesex, UK) was used in this study. Cells were routinely passaged and maintained in RPMI media (Lonza, UK), supplemented

with 15% FBS at 37^0C in a humidified environment of 5% CO_2 in air. Cell viability was assessed using the Trypan blue exclusion method on a haemocytometer, and experiments were only performed when cell viability was >95%.

2.10 Epi-Fluorescence Microscopy

Human Umbilical vein endothelial cells (HUVEC) cells were cultured for 24 hours on glass cover slips and fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 10 minutes at room temperature. Then HUVEC were permeabilised with 1% triton-100 in PBS for 5 minutes and blocked with blocking buffer (5% goat serum and 0.1% triton x-100 in PBS) for 1 hour at room temperature. Then the cells were labelled with mouse anti-CD62P (Abcam, UK, Cat: NB100-65392) and rabbit ant-CD62E (Abcam, UK, Cat: ab18981) in primary solution (1:1 ratio of blocked buffer and PBS) with 1:300 ratio. Following incubation overnight in the dark at 4°C, the HUVECs were stained with secondary antibody Alexa-568 anti-rabbit IgG (Invitrogen, UK, Cat: A11011) and Alexa-647 anti-mouse IgG (Invitrogen, UK, Cat: A21235) simultaneously, in primary solution at 1:500 ratio. The cells were stained with one drop of DAPI (diamidino-2-phenylindole) mounting solution (Vector Shield Laboratories, UK, Cat: H1200) overnight at 4°C and covered from light ready for EPI microscopy. For Epi-fluorescent microscopy the Zeiss imager Z1 microscope was used and 40x lens was obtained the images from.

2.11 Western Blotting

Washed platelet samples were treated with thienopyridines as described in section 2.0. Following a 30 minute incubation, the samples were stimulated with 10µM ADP and immediately centrifuged for 5 min at 1500 g. The supernatants were discarded and the

platelet pellets were resuspended in 200 µl Pierce ™ radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific), containing a 1:1000 dilution of both phosphatase and protease inhibitors to protect platelet proteins from denaturation. The samples were vortexed and placed on ice for 30 minutes. Vortexing was performed every 5 minutes until the end of the 30 minutes duration. Subsequently, mixtures were put in an ultrasonic bath- Grant- XUBA3 (sonicator) for 3 minutes to ensure the breakdown of platelet membranes. Samples were then centrifuged at 13500 g for 20 minutes and the supernatants (platelet lysates) were then frozen at -20°C until further analysis.

2.11.1 Protein Assay

Protein assays were performed using the BCA Protein Assay (Thermo Scientific) to quantify the proteins in the platelet lysates prior to Western blotting to normalize lane loading. The BCA assay is a colorimetric technique, which depends on cupric ions (Cu2+) reduction by peptide bonds in proteins. The amount of reduced Cu2+ ions is directly proportional to the amount of proteins existing in the sample. When *the BCA* reagent binds to Cu2+ ions, a purple-coloured substance is formed and absorbs the light at a wavelength of 562 nm.

Bovine Serum Albumin (BSA) protein standards were prepared by diluting the BSA standard samples (2 mg/mL) (Thermo Scientific, 23209) in RIPA buffer at concentrations of 2, 1.6, 1.2, 0.8, 0.4 ,0.2 and 0 mg/ml. Platelet lysates were diluted twice with RIPA buffer at ratios of 1:1 and 1:2 in order to ensure that final absorbance values following BCA assay were within the range of the standard curve.

BCA reagents A & B were mixed in a ratio of 1:50. A 200µL volume of the mixed solution was added to a 96 well plate with 10µL of the protein samples or standards. The 96 well plates were incubated at 37^{o}C and absorbance was recorded after 30 minutes at 562 nM using a Bio-Tek ELISA plate reader (Winooski, VT, USA). The sample protein concentrations were calculated from the standard curve that was generated automatically by plotting the average of loaded BSA standard at 562 nm against the known concentration in mg/ml.

2.11.2 Gel Electrophoresis

Following protein extraction and determination of protein concentration, samples were loaded according to protein concentration (12µg per sample in a maximum 30µL total volume used). An 8µL volume of Precision Plus proteins Dual standards markers (Bio-Rad, UK), was prepared alongside the samples, as a reference for the molecular weight of migrating proteins. Loading buffer was made up by diluting nine part of 4x Laemmli Sample Buffer (Bio-Rad, UK) in one parts of 2-mercapto-ethanol. Each platelet lysate sample was then diluted in loading buffer at a ratio of 1:3. Samples were boiled at 100◦C for 10 minutes, in order to reduce disulphide bonds and facilitate protein denaturation, followed by immediate transfer to ice.

The resolving gel was prepared by using TGX and TGX Stain-Free Fast Cast kit (Bio-Rad, USA) where a 1:1 ratio of resolver A and B were mixed together to make the resolver solution (3ml each to do 1mm glass plate). This was followed by addition of 30 µL of 10%

ammonium persulfate (APS) and 3 µL of N,N,N,N'-tetramethylenediamine (TEMED) per cassette, which form a polymer network/mesh that catalyzes the polymerization of the reaction. The stacking gel was prepared by mixing a stacker A and B in a 1:1 ratio followed by addition of 2 µL TEMED and 10 µL APS per cassette. The resolving gel and stacking gel were assembled in a Bio-rad glass cassette and a comb was inserted over stacking gel solution. The gel was left for 1-2 hours to allow time for solidification.

Following gel formation, the glass plate holding the gel was placed in the electrophoresis chamber. Running buffer was prepared (25 mM Tris, pH 8.3, 190 mM glycine, 0.1% SDS in dH20) and used to completely cover the gel. The leakage of running buffer through the stands holding the gel was prevented. The chamber was then filled with the running buffer until the level reached the first green bar in the stand holding the glass plates. The comb was removed gently from the gel and 20 µL of Precision Plus Protein TM Dual Colour standards was loaded into the first well of the gel, then 20 µL of each sample was loaded in the following wells. The gel was run at 400 MA, 100 V for 10 minutes until the bromophenol blue dye, included in the sample buffer, reached the interface between the stacking and the resolving gels. Voltage was subsequently increased to 200V for approximately 30 minutes until the bromophenol blue dye reached the end of the resolving gel.

2.11.3 Blotting

Once the protein has been separated by gel electrophoresis, proteins were transferred to a nitrocellulose membrane (TransBlot Turbo mini-size nitrocellulose) (Bio-Rad, USA).

The blotting was performed by semi-dry method using the Trans-Blot Turbo Blotter (BioRad, USA). Two filter papers were immersed in transfer buffer before using to create a sandwich with the gel and the nitrocellulose membrane between them. The sandwich was placed into the TransBlot system and run at 25 Volts for 7 minutes for transfer to take place.

2.11.4 Blocking

A blocking stage was needed to prevent any non-specific binding between the primary antibody and non-target proteins on the blot. Bovine serum albumin (BSA) (Sigma-Aldrich, UK) 2% in TBST was used to block the membrane for 1 hour at R.T. on a shaker.

2.11.5 Detection

For detection of target proteins, primary antibodies (Ab) (Table 2.2) were diluted in blocking buffer (1:1000) and incubated with the membrane overnight at 4°C on a shaking machine. The following day, the primary Ab solution was discarded and the membrane was washed 3 times with Tris-buffered saline with tween 20 (TBST) for 5 minutes per wash on shaker at R.T. The membrane was then incubated with a secondary Ab, anti-rabbit IgG-Horseradish Peroxidase (HRP) (Table 2.2) for 1 hour at R.T on a shaker. The membrane was then washed 3 times again as before prior to exposure to enhanced chemilluminescence (ECL). The substrate used for this is Piece ECL Western

Blotting substrate (Thermo Fisher, UK). It was prepared by mixing reagent A with reagent B in 1:1 ratio and 1 ml of the mixer was added to cover the membrane for 5 minutes at R.T without shaking. Finally, the membrane was analysed using the BioRad imaging system where the chemiluminescence produced from enzymatic reaction between HRP on the secondary Ab and the ECL substrate, is proportional to the amount of protein in the membrane (Dimechev, 2012).

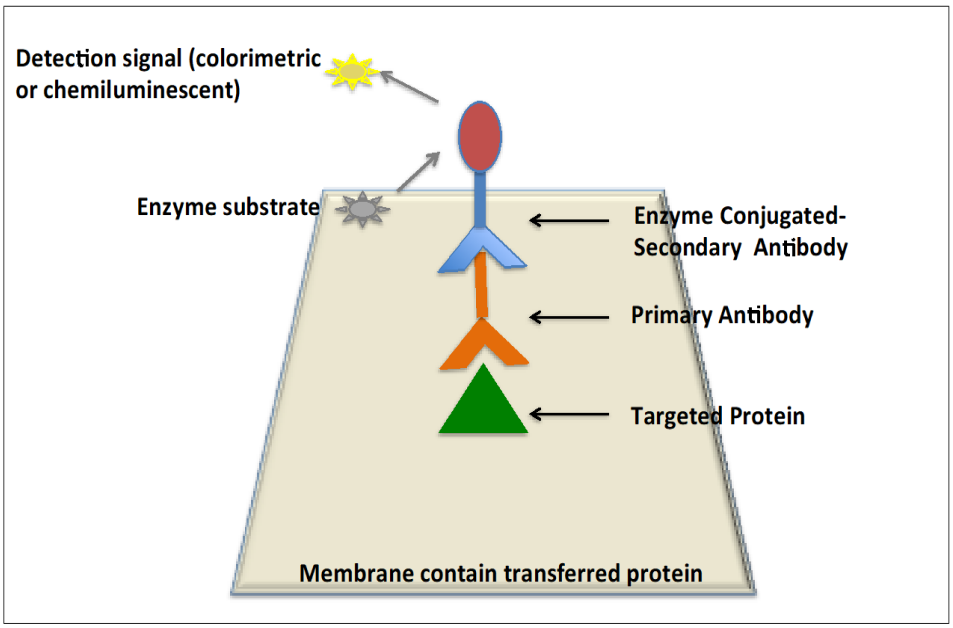


Figure 2.1 Detection and quantification of target proteins. Target proteins, transferred to nitrocellulose membranes, are bound by primary antibodies, which are recognized by enzyme-conjugated secondary antibodies. The enzyme, such as HRP, participates in a chemical reaction when a specific substrate is added, resulting in the emission of a detectable signal. The light signal can be detected by a camera, enabling the semi-quantification of the target proteins present on the nitrocellulose membrane (adapted from Leinco Technology, 2018)

Table 2-2 Antibodies lists that used for western blot.

Antibody	Company supplier	Catalogue Number	Dilution
VASP	Cell Signalling Technology, UK	3132	1:1000
p-VASP	Cell Signalling Technology, UK	3114S	1:1000
Akt	Cell Signalling Technology, UK	9272S	1:1000
p-Akt	Cell Signalling Technology, UK	9271S	1:1000
PLCγ2	Cell Signalling Technology, UK	3872	1:1000
p-PLCγ2	Cell Signalling Technology, UK	3874S	1:1000
cPLA2§	Cell Signalling Technology, UK	2832S	1:1000
p-cPLA2	Cell Signalling Technology, UK	2831	1:1000
Anti-Rabbit Igg-HRP Linked Ab	Cell Signalling Technology, UK	7074S	1:1000

VASP: Vasodilator-Stimulated phosphoprotein, Akt also known protein kinase B, PLCγ2 : phospholipase C gamma 2, cPLA2: cytosolic phospholipase A2.

2.11.6 Nitrocellulose Membrane Stripping and Re-Probing

Nitrocellulose membrane stripping was performed to enable re- probing of the membrane using alternative primary antibodies. To strip, membranes were incubated 15 minutes at RT with Restore Western Blot Stripping Buffer (Thermo, Rockford, USA). Nitrocellulose membranes were washed three times, for 5 minutes each time with TBST and then re-blocked by incubating with blocking buffer for 1h at RT on a shaker. When blocking buffer has been removed, the membrane was incubated with anti-β-actin (New England Biolabs) or an antibody to the unphosphorylated form of the protein under investigation (Anti-total VASP, Anti-total Akt, Anti-total PLCγ2, anti-total cPLA-2) (New England Biolabs)) at 4°C on the shaker, overnight. The primary and the secondary antibodies were prepared by diluting with 2% BSA (1 in 1000 dilution). Thereafter, the membrane was washed with TBST three times and incubated with the secondary antibody and then with the chemiluminescent substrate as described previously, and blots were visualised using the imaging system.

Densitometry was applied for quantifying the proteins after each visualization step. Where appropriate, all phopshorylated forms of proteins investigated were normalized either to their respective total protein levels or to β-actin. All antibodies used for western blotting analysis were purchased from New England Biolabs (Hertfordshire, UK).

2.12 Statistical analyses

All data normalisation was performed using Microsoft Excel. Statistical analysis was performed using GraphPad PRISM version 6.0. Specific statistical analysis applied to data sets is explained within the specific experimental chapters. A $p<0.05$ is considered statistically significant.

Chapter 3: Novel Thienopyridines as modulators of platelet function.

Chapter 3: Novel Thienopyridines as modulators of platelet function.

3.1 Introduction

Platelet hyperactivity is often a major contributory factor in the development of disorders such as acute coronary syndrome (ACS) and also stroke due to acute arterial thrombosis. Myocardial infraction (MI) and stroke are currently the two most common causes of morbidity in the developed world (Zetterberg and Svensson, 2016). Thus, the ability to control platelet activity and reduce adverse arterial thrombus formation is a critical tool in modern clinical practice.

Due to the importance of P2Y12 in platelet activation, P2Y12 inhibitors have been developed which bind to the receptor, thereby blocking the binding of ADP. Indeed, a combination treatment of a P2Y12 inhibitor and a COX-1 inhibitor, most commonly clopidogrel and aspirin (acetylsalicylic acid, (ASA)) results in reduced cardiac events in patients with ACS and patients having undergone percutaneous coronary intervention (stents) (Oh et al., 2016). Prior to FDMA approval, clopidogrel was shown to reduce the risk of death/MI and stroke in the CURE (Clopidogrel in Unstable angina to prevent Recurrent Events) and CREDO (Clopidogrel for the Reduction of Events During Observation) trials, and it was concluded that clopidogrel and ASA treatment had long-term benefits (Oh et al., 2016; Emmons and Taylor, 2007; Jain et al., 2016; Li et al., 2016). However, ASA and clopidogrel combination treatment is usually only recommended for a maximum of 12 months due to the potential for gut damage and bleeding. Of those patients that are suitable for treatment, approximately 4%-30% will be classed as 'non-

responders' (Saab et al., 2015). Currently there are several proposed reasons for clopidogrel poor/no response which include genetics (CYP2C19*2 loss of function allele, $P2Y_{12}$ receptor gene polymorphism), drug interactions, (e.g. Paclitaxel, Statins, Ca^{2+} channel blockers), patient body mass index and co-morbidities such as Diabetes, intestinal conditions and impaired renal function (Damman et al., 2012; Bouman et al., 2010; Fontana and Reny, 2008; T. Gremmel et al., 2011; Hulot et al., 2006; Kim et al., 2016; Angiolillo et al., 2009; Shinoda et al., 2016).

Prasugrel and cangrelor are newer thienopyridine-type drugs with an improved efficacy but at the cost of an increased bleed risk, with the JUMBO-TIMI (Joint Utilization of Medications to Block Platelets Optimally – Thrombolysis in Myocardial Infarction) study finding significantly more bleeding events in patients taking prasugrel compared to patients taking clopidogrel (Nikolaus and Robert, 2012; Damman et al., 2012). However, in a randomised trial, Kim *et al*. found that prasugrel, was able to overcome the poor outcome for the CYP2C19*2 loss-of-function allele seen in some clopidogrel non-responders (Kim et al., 2016). This suggests that refinement of this family of compound may be useful in these patients.

More recently, the synthesis of structurally related thienopyridines have been reported and found to have potent phospholipase C (PLC) inhibitory activity, while under investigation for their anti-cancer properties. To date, the anti-platelet properties of these thienopyridine derivatives has not investigated on platelets.

Our collaborators at the University of Auckland, New Zealand, synthesized six novel thienopyridine derivatives, displaying various sized and functionalized cycloalkyl rings as well as various substitutions on the phenyl ring (Table 3.1). All compounds were obtained as pure solids after recrystallisation from methanol and their structures were confirmed using NMR and mass spectrometry.

The aim of this chapter was to assess the anti-platelet activity of these novel thienopyridines in comparison with the standard treatment, clopidogrel, by analysing platelet activation and platelet function following treatment with the compounds and stimulation with a platelet agonist. This would determine whether these novel derivatives have potential for future development for use in patients who have shown resistance to the standard anti-platelet drugs.

3.2 Methods

3.2.1 Ethics

See section 2.2 for information on ethical approval and patient recruitment.

This section of the study involved a total of eleven participants (n=3 male, n=8 female, age range 20-38). Samples from some participants (chosen at random) were used for analysis of multiple markers of platelet activation and function.

3.2.2 Sample collection and platelet-rich plasma purification

Blood was collected and platelet-rich plasma purified as described in section 2.3.

3.2.3 Thienopyridine treatment

Thienopyridine treatments were performed as described in section 2.5. Clopidogrel active metabolite (Sigma, UK), thienopyridines or vehicle control (DMSO) were used at a final concentration of 10 μM for 30 min at 37 °C. ASA (Sigma, UK) was used at a final concentration of 30 μM.

3.2.4 Platelet activation analysis

Platelet activation was assessed via flow cytometry analysis of CD62P and PAC1 using a PE-conjugated anti-human CD62P and a FITC conjugated PAC-1 (both (BD Biosciences, UK) as described in section 2.6 ADP (Labmedics, UK) was used as the agonist at a concentration of 10 μM).

3.2.5 Light transmission aggregometry

Platelet aggregometry was performed on drug-treated platelets as described in section 2.7 ADP and Collagen were used as agonists at final concentrations of 10 μM and 1 μg/ml (Labmedics, UK) respectively.

3.2.6 Platelet-leukocyte aggregate analysis

Platelet leukocyte aggregates were analysed as described in section 2.6 PE-conjugated anti-human CD45 and FITC-conjugated anti-human CD42b (both BD Biosciences, UK) were used to identify leukocytes and platelets respectively in while blood.

3.2.7 Statistical analysis

Student t-tests for paired data were used to compare CD62P, PAC1 and MaxA values in drug-treated samples with that in control samples. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$ when comparing with control.

ψ represents $p < 0.05$, ψψ represents $p < 0.01$, ψψψ represents $p < 0.001$ when comparing drug-treated samples with clopidogrel treated samples by paired t-test.

In ASA synergy experiments, t-tests for paired data were used to compare thienopyridine-only treated samples or ASA-treated samples with thienopyridine-ASA combination treated samples, and also each thienopyridine-ASA combination treated sample with clopidogrel-ASA treated samples. ϕ represents differences between combination treated samples and clopidogrel+ASA treated samples.

3.3 Results

3.3.1 Novel thienopyridines inhibit ADP-stimulated platelet activation

The expression of CD62P (P-selectin) along with PAC1 binding as markers of platelet α-granule secretion and fibrinogen receptor activation respectively were analysed in PRP samples following treatment with the thienopyridine compounds. The PRP was stimulated with ADP in order to induce platelet activation and hence expression of the two markers. Samples were treated with clopidogrel (active metabolite), thienopyridine or vehicle control for 30 min prior to ADP stimulation. All novel thienopyridines resulted in a significant decrease in CD62P expression when compared to ADP-stimulated controls (Fig 3.1A). When PAC1 binding was analysed, all six thienopyridines resulted in inhibition, while clopidogrel was unable to produce the same effect. More interestingly, three of the six thienopyridines (DJ0171, DJ0097 and DJ0199) were able to inhibit the expression of CD62P and PAC1 binding to a greater degree than clopidogrel (Figs 3.1A & 3.1B). In the case of PAC1 binding, this was also true of DJ0206, DJ00221 and DJ0081. Thienopyridine DJ0199 appeared to be the most superior compound at causing this effect. Taken together, these data show that the novel thienopyridines inhibited platelet activation in the presence of ADP and, under these conditions, were more effective than clopidogrel.

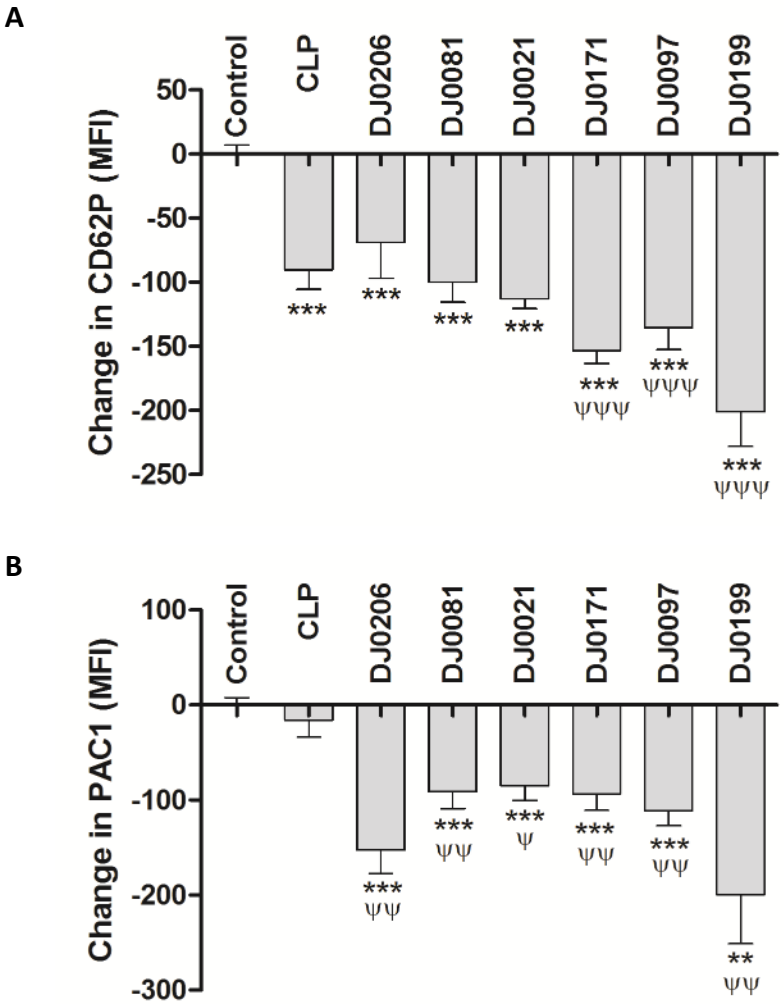


Figure 3.1 Change in CD62P (A) and PAC-1 (B) expression in ADP-stimulated PRP following thienopyridine treatment was assessed by flow cytometry. Change in expression (i.e. inhibition) relative to control was determined. Data are presented as Mean ± SEM of four independent blood donors, n = 4. Statistical analysis was performed using the Student's t-test for paired data to determine differences from control and from clopidogrel-treated samples. Significant differences from control (*) and clopidogrel (ψ) are indicated.

3.3.2 Novel thienopyridines inhibit ADP-stimulated platelet aggregation in PRP

After assessing platelet activation, light transmission aggregometry (LTA) was used to assess ADP-stimulated platelet aggregation in PRP following 30 min treatment with 10 µM of clopidogrel or the novel thienopyridines. This allowed the assessment of platelet function. Treatment with all six novel thienopyridines resulted in a significant reduction in maximum aggregation when compared to vehicle-control treated PRP (Fig 3.2). A paired t-test, to compare MaxA values after novel thienopyridine treatment with that following clopidogrel treatment, revealed that DJ0021, DJ0171 and DJ0199 caused a significantly greater inhibition of aggregation ($p = 0.0009$, $p = 0.0124$ and $p = 0.0016$ respectively) (Fig 3.2).

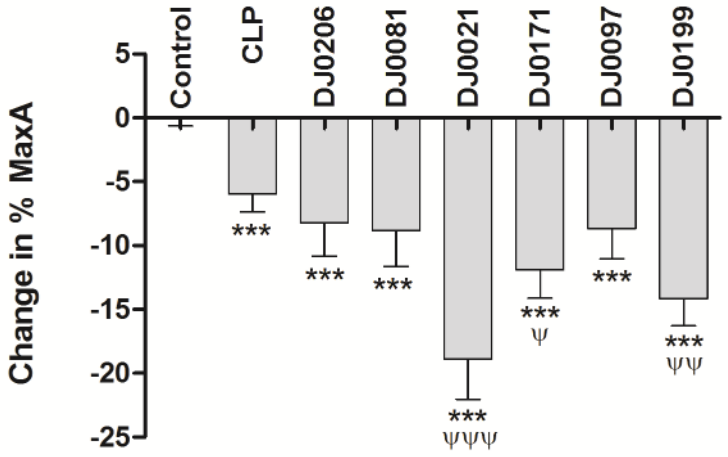


Figure 3.2 Change in maximum aggregation (MaxA) in ADP-stimulated PRP following thienopyridine treatment was assessed by light-transmission aggregometry. Change in aggregation (i.e. inhibition) relative to control was determined. Data are presented as Mean ± SEM of four independent blood donors, where n = 7. Statistical analysis was performed using the Student's t-test for paired data to compare each drug-treated sample with the control and with clopidogrel-treated samples. Significant differences from control (*) and clopidogrel (ψ) are indicated.

3.3.3 Novel thienopyridines inhibit collagen-stimulated platelet aggregation in whole blood.

Although thienopyridines block ADP-induced aggregation, an effect on collagen-induced activation should also be observed as the secondary wave of platelet aggregation caused by dense-granule-derived ADP inhibition. Indeed, the novel compounds inhibited collagen-induced aggregation of PRP, but to a lesser degree than ADP-induced aggregation (Fig 3.3). Interestingly, platelet aggregation in clopidogrel-treated samples was not significantly different from aggregation in the untreated samples, whilst the novel thienopyridines appeared more effective at inhibiting collagen-induced aggregation than clopidogrel, with the exceptions of DJ0206.

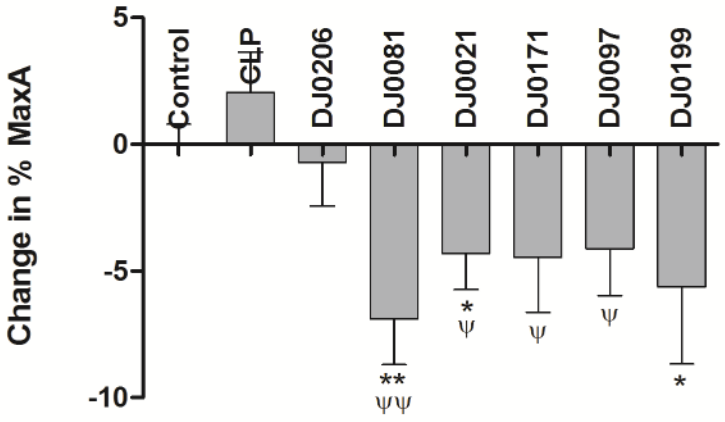


Figure 3.3 Change in maximum aggregation (MaxA) in collagen-stimulated PRP following thienopyridine treatment was assessed by light-transmission aggregometry. Change in aggregation (i.e. inhibition) relative to control was determined. Data are presented as Mean ± SEM of four independent blood donors, where n = 3. Statistical analysis was performed using the Student's t-test for paired data to compare each drug-treated sample with the control and with clopidogrel-treated samples. Significant differences from control (*) and clopidogrel (ψ) are indicated.

3.3.4 Novel thienopyridines inhibit ADP-induced platelet-leukocyte aggregate formation

Following activation, platelets may adhere to local leukocytes (monocytes and neutrophils) via platelet CD62P binding with P-selectin glycoprotein ligand-1 (PSGL1) on the leukocyte surface. Platelet-leukocyte aggregates are considered a reliable marker of pro-thrombotic state. Whole blood samples were pre-treated with clopidogrel, thienopyridine or vehicle control for 30 min prior to ADP stimulation. Samples were double stained with the platelet marker CD42b and the leukocyte marker CD45 and analysed using flow cytometry. Platelets were identified in the whole blood sample (Fig 3.4A) by expression of CD42b and gated (Fig 3.4B). CD45-positive events within the platelet gate were identified (Fig 3.4C). All novel thienopyridines, resulted in a significant decrease in the percentage of platelet-leukocyte aggregates when compared to ADP-stimulated controls (Fig 3.5). Statistical analyses to compare percentage of aggregates in the thienopyridine-treated samples with the clopidogrel-treated samples revealed a significant difference following treatment with DJ0097.

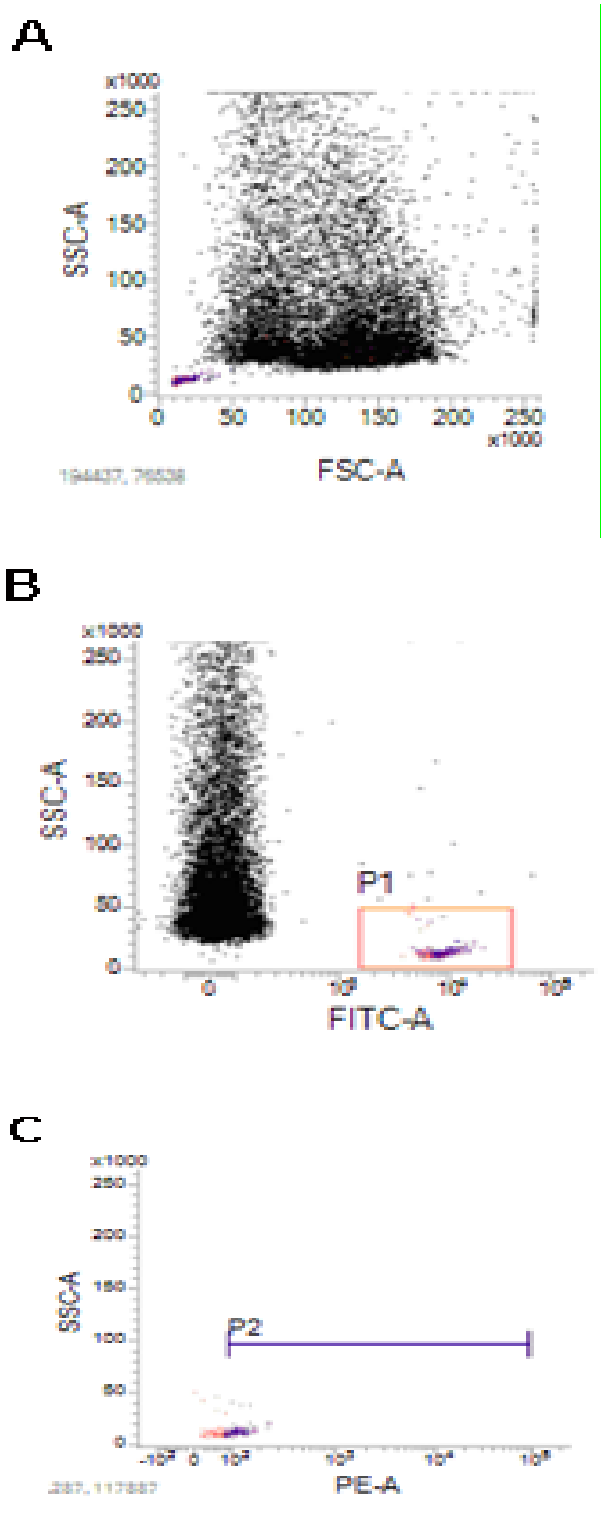
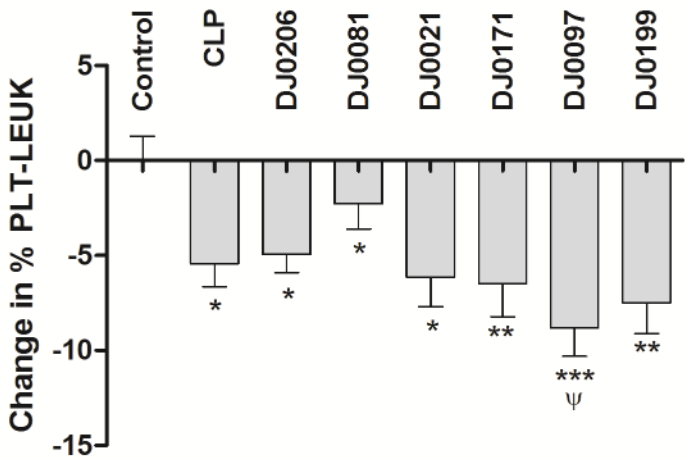


Figure 3.4 (A) Whole blood was analysed by forward scatter (FSC) and side scatter (SSC). (B) The platelet population was identified by expression of CD42b and gated (P1). (C) CD45 positive events within the platelet gate (P1) were identified (P2).



tes within the ADP-stimulated whole blood samples following thienopyridine treatment was assessed by flow cytometry. Change in CD42b+/CD45+ events (i.e. inhibition) relative to control was determined. Data are presented as Mean ± SEM of four independent blood donors, where n =4. Statistical analysis was performed using the Student's t-test for paired data to determine differences from control (*) and from clopidogrel-treated samples.

3.3.5 Novel thienopyridines show synergy with Aspirin

Management of ACS or stroke patients often involves the use of clopidogrel in combination with ASA based on a duel-hit hypothesis, whereby platelet function is inhibited via simultaneous inhibition of the $P2Y_{12}$ receptor and the COX-1 enzyme. We investigated the synergistic action of the novel thienopyridines and ASA, using LTA to assess platelet function.

At the concentrations used in this study, all thienopyridines caused greater inhibition of platelet aggregation when compared with the inhibition caused by ASA alone (Fig 3.6) (represented by *). This was also true of clopidogrel. When thienopyridine-combination treatments were compared with the respective thienopyridine only treatments, all showed synergy with ASA, with the exception of DJ0206 and DJ0021 **3c** and **3b** (represented by ψ). However, these thienopyridines were shown to have a significant inhibitory effect when used in isolation. An important facet of this work was to determine whether any of these novel thienopyridines were more potent when used in combination with ASA, than the combination of clopidogrel and ASA. It was demonstrated that all compounds with the exception of DJ0206 showed significantly greater activity than clopidogrel when used in combination with ASA (represented by ϕ).

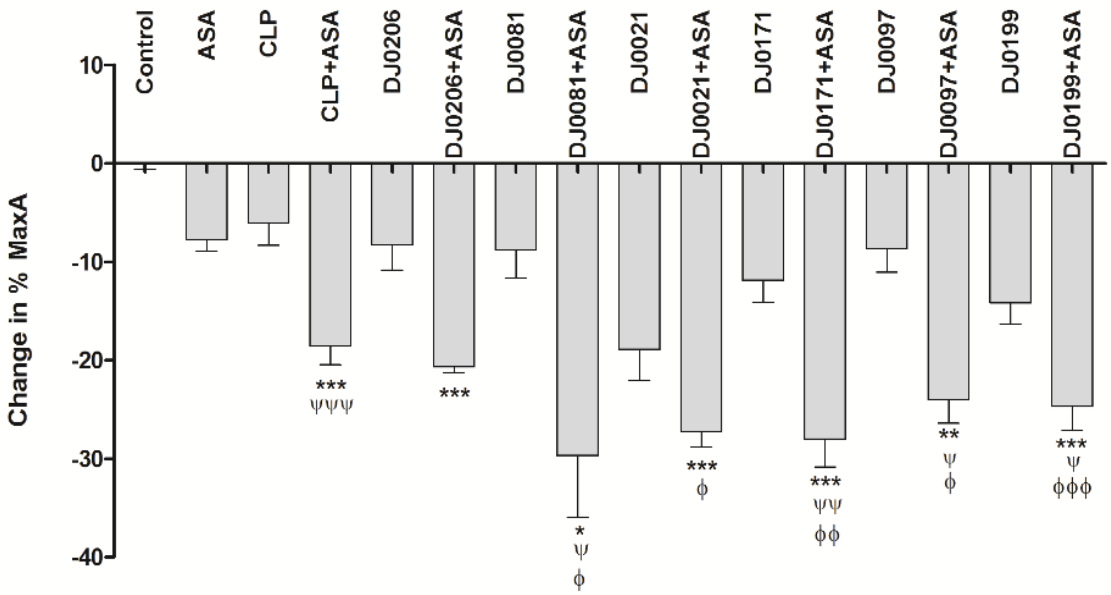


Figure 3.6 Change in maximum aggregation (MaxA) in ADP-stimulated PRP following thienopyridine treatment in the presence or absence of ASA was assessed by LTA. Change in aggregation (i.e. inhibition) relative to control was determined. Data are presented as Mean ± SEM of seven independent blood donors, n=7. Statistical analysis was performed using the Student's t-test for paired data to compare each

3.4 Discussion

Although platelet inhibitors such as clopidogrel and, more recently prasugrel and ticagrelor are currently used in clinical practice, the continued platelet hyperactivity in some patients taking these drugs highlights a need for continued refinement of this class of drugs (Franchi and Angiolillo, 2015). The present study provides a significant contribution to the literature on $P2Y_{12}$ inhibitor therapy by reporting on the use of novel thienopyridine derivatives and their greater activity when compared to clopidogrel.

All six novel compounds significantly inhibited expression of both CD62P and PAC1 when compared to ADP-stimulated controls. When looking at the inhibition of PAC1, all the compounds had increased activity when compared with clopidogrel, while only DJ0171, DJ0097 and DJ0199 showed greater activity in the inhibition of CD62P. Although both CD62P and PAC1 reflect platelet activation, CD62P is expressed upon alpha-granule release (Blair and Flaumenhaft, 2009), while PAC1 binds to activated GP IIb/IIIa (fibrinogen receptor) (Shattil et al., 1985). CD62P release and fibrinogen receptor activation are not necessarily simultaneous events during the process of platelet activation (Y.A and A.V, 2003). It is therefore important to analyse more than one marker of platelet activation when examining the effects of anti-platelet drugs. In the present study, clopidogrel had a very small effect on PAC1 binding, with all six novel thienopyridines showing greater efficacy. This difference is likely to be highly important *in vivo*.

Aggregometry was used in this study to assess platelet function. All the thienopyridines were found to inhibit ADP-induced platelet aggregation, with DJ0021, DJ0171 and DJ0199 showing greater efficacy than clopidogrel. Furthermore, collagen-induced

aggregation was also hindered to some degree following derivative treatment, highlighting the importance of $P2Y_{12}$ in secondary activation from dense-granule-derived ADP. LTA is a gold standard measure of platelet function and is included in the majority of studies focusing on anti-platelet drugs (Bouman et al., 2010; Armstrong et al., 2011; Valenti et al., 2015). However, strong platelet activation can occur without significant end-point aggregation and, activated platelets even with poor aggregation will still exert a systemic effect, increasing inflammation and stimulating further platelet activation (Vistoli et al., 2014). Therefore, it is critical to monitor both activation status and aggregation to assess the global effects of anti-platelet drugs.

Platelet-leukocyte aggregate formation has been shown to be increased in patients with ACS (Mark I. Furman et al., 1998; Ott et al., 1996; Sarma et al., 2002) and has been suggested as a monitoring tool for risk of MI in these patients (M. I. Furman et al., 2001). Platelet-leukocyte aggregates have also been suggested to be a superior marker of platelet activation when compared with CD62P expression (Michelson et al., 2001). The data presented here shows that all six novel thienopyridine derivatives, as well as clopidogrel, were able to inhibit ADP-induced platelet-leukocyte aggregate formation. This is in agreement with similar studies that report decreases in platelet-leukocyte aggregate formation following clopidogrel, prasugrel or cangrelor treatment, both *in vitro* and *in vivo*(Thomas Gremmel et al., 2013; Harding et al., 2006; Klinkhardt et al., 2003; Klinkhardt and Harder, 2005).

Exploring synergy of the novel thienopyridine derivatives with ASA revealed that platelet aggregation was inhibited to a greater extent when any of the novel compounds were

used in combination with ASA, when compared with ASA or derivative alone. The exception to this was DJ0021 which although appeared to show greater efficacy when used as part of the combination treatment, statistical analysis proved this to be insignificant. More interestingly, when combined with ASA, all thienopyridines, with the exception of DJ0206, showed a greater inhibitory effect than clopidogrel+ASA. These data support results which demonstrate a superior platelet-inhibitory effect of clopidogrel+ASA compared to either treatment alone (Moshfegh et al., 2000; Serebruany et al., 2003). Armstrong *et al*. studied the combination of prasugrel and ASA on platelet activation *in vitro* and reported that ASA did not significantly increase the inhibitory effect of prasugrel (Armstrong et al., 2011). It was proposed that in the presence of strong $P2Y_{12}$ receptor blockade, ASA does not provide any benefit. However, our novel derivatives were extremely effective at $P2Y_{12}$ receptor blockade when used in isolation, and yet were enhanced by ASA. There is a lack of literature documenting the effects of prasugrel/cangrelor and ASA *in vivo*, but it appears that ASA-thienopyridine synergy is $P2Y_{12}$ inhibitor-specific.

It is clear that despite being structurally related molecules, some thienopyridines have greater activity than others. Indeed, prasugrel, clopidogrel and cangrelor all affect platelet function to varying degrees in different patient groups (Harrington et al., 2009; Jia et al., 2015; Roe et al., 2012; Wiviott et al., 2006). These novel derivatives follow this pattern and although this study has shown that all of them have the ability to inhibit activation and aggregation to some degree, some demonstrated greater activity. Taken together, these data show that DJ0171 and DJ0199 are very effective platelet inhibitors. They are both more effective than clopidogrel at inhibiting the expression of CD62P and

PAC1 in response to ADP stimulation. They are more effective at inhibiting platelet aggregation both alone and in combination with ASA. This global reduction in both platelet aggregation and activation highlights these molecules as worthy of further investigation to determine their potential as $P2Y_{12}$ inhibitors in the clinical setting such as western blot to test platelet signaling.

When comparing the molecular structures of the tested thienopyridines the most consistently active compound, DJ0171, has a strong similarity to clopidogrel with both containing a 2-chlorophenyl moiety linked to a larger heterocyclic group. This similar motif of a 2-substituted phenyl group is also found in the commonly used drug prasugrel. The active compound DJ0199 interestingly does not contain a 2-substituent on its phenyl ring. This suggests that alternative substitution patterns in these series of compounds can still lead to viably active compounds.

Of course, when considering any drug aimed at inhibiting platelet function, it is important to consider over-effectiveness, with risk of bleeding becoming an issue. Indeed, the superior activity of prasugrel over clopidogrel has also been associated with increased risk of bleeding in some studies (Damman et al., 2012; Nikolaus and Robert, 2012; Wiviott et al., 2007). It will be important to determine whether our derivatives are also associated with increased bleed risk *in vivo*.

In conclusion, the six derivatives tested all possessed anti-platelet activity, showing inhibitory effects on ADP-induced CD62P expression and PAC1 binding, platelet-leukocyte aggregate formation and aggregation. The work highlights the potential of

these compounds as alternative treatments in platelet hyperactivity-associated disorders.

Chapter 4: Examining the mechanism of action of novel Thienopyridines

Chapter 4: Examining the mechanism of action of novel Thienopyridines.

4.1 Introduction

Platelets are normally in a resting state and become activated at the site of vascular damage. This follows exposure to adhesive proteins expressed by endothelial cells such as collagen and VWF or by release of agonists such as ADP, Thrombin or TXA2 from neighbouring platelets that are already in an activated state (Li et al., 2010). As mentioned previously in section 1.5, platelet signalling involves three stages; (1) Interaction between the agonist and its corresponding receptor (2) activation of specific signalling pathways that may include downstream signalling for more than one extracellular receptor, and (3) inside- outside signalling (Bye et al., 2016). Many of the initial signalling pathways converge to activate the same set of protein kinases, yet further upstream, binding of specific agonists to specific receptors initiates a distinct set of signalling events (Bye et al., 2016). Thus, understanding this network of signals is important for investigating platelet function and developing new antiplatelet drugs for cardiovascular disease patients. The current treatments for cardiovascular patients are targeting the P2Y12 receptor due to its importance in platelet activation. ADP is a natural agonist for P2Y12 and it is stored in dense granules of platelets that release ADP as a positive feedback when the platelets have been stimulated by other agonists such as Thrombin, Collagen or TXA2. Therefore, the P2Y12 receptor plays a pivotal role in platelet activation and aggregation.

Studying the key regulators of intracellular platelet signalling such as cytosolic phospholipase C (cPLC), Phosphatidylinositol 3-Kinases (PI3K) and protein kinase C (PKC), and also key kinases downstream of these such as PLCγ2, Vasodilator stimulation Phosphoprotein (VASP), Akt (Known also Protein Kinase B) and cPLA2, in response to platelet inhibitors is important to fully understand the global effects on platelet activation and function (Gibbins et al., 2004).

VASP signaling is extremely useful to measure in any investigation of platelet function/inhibition (Hezard et al., 2005). It is a substrate cyclic adenosine monophosphate- (cAMP) and guanosin monophosphate (GMP)-dependent protein kinase (Pampuch et al., 2006) involved in the inhibition of agonist-induced platelet aggregation. Upon stimulation of P2Y12, there is suppression of adenyl cyclase via Gi-protien, and inhibition of cAMP production. Therefore, ADP inhibits PGE1-induced VASP phosphorylation. Once a P2Y12 inhibitor is added into the system, there is a lack of inhibition to PGE1-induced VASP phosphorylation. Therefore, the better the P2Y12 inhibitor, the more VASP phosphorylation is observed (Aleil et al., 2005). VASP analysis was included in this study to investigate the effect of novel thienopyridine treatments which may work as specific blockers for P2Y12 receptor through a similar mechanism to clopidogrel, another member of thethienopyryidine family.

Another signaling molecule analysed in this study is Akt which is a signal molecule downstream of the PI3K isoform (Kim et al., 2004). Thrombin has been reported to increase the activity of Akt1 by phospholipase β2-dependent through Ca^{2+} dependent PKc subtype (Kroner et al., 2000). It has been reported that stimulation of the G_i signaling

pathway by ADP leads to activation/phosphorylation of Akt in platelets (Kim et al., 2004). However, although P2Y12 was once thought to be critical for Akt activation, more recent data demonstrated that Akt phosphorylation can occur via both ADP/$P2Y_{12}$/G_i-dependent and ADP/$P2Y_{12}$/G_i-independent mechanisms (Xiang et al., 2010). Nevertheless, assessment of the degree of Akt phosphorylation in the presence of P2Y12 inhibitors would be a useful indicator of efficacy – higher platelet activity should be reflected by greater Akt phosphorylation.

Cytosolic phospholipase A2 (cPLA2) is another important signaling molecule thought primarily to act downstream of the Thrombin receptors PAR-1 and PAR-4 (and to a lesser extent GPVI) (Shankar et al., 2006). Phosphorylation of cPLA2 results in release of arachidonic acid (AA), ADP release from dense granules and ultimately platelet aggregation. However, these is some evidence to suggest that activation of cPLA2 may in fact occur after P2Y2 stimulation. Cristofer et al (1989) have demonstrated that ADP treatment of platelets can stimulate Na+ /H+ exchange, increasing cystolic pH, which in-turn leads to an increase in Ca^{2+} and activation of cPLA2. Moreover, it has been reported that Ethyllisorpopylamilorode, an inhibitor of Na+ /H+ exchange decreases pH and blocks ADP-induction of cPLA2 (Puri et al. 1998). A better understanding of the effect of P2Y12 inhibitors on activation status of cPLA2 would be a useful contribution to knowledge.

PLCγ2 is one isoform of the PLC family which is important in platelet activation (Mangin et al., 2003). PLCγ2 is activated downstream of immunoreceptor tyrosine-based activation motif (ITAM)-bearing adhesion receptors, namely GPVI-FcRc/FccRIIA and C-

type lectin receptor 2 (CLEC-2). As a result, collagen is the platelet agonist mainly responsible for activating PLCγ2. Collagen and fibrinogen toegther can also activate PLCγ2 via α2β1 and $\alpha_{IIb}\beta_3$ respectively (Bye et al., 2016). Although there is evidence that ADP itself can activate PLCγ2, albeit with weaker effect that collagen, this has been shown to be via binding of ADP to P2Y1 paired with G_q, (Kunapuli et al., 2003; Gibbins et al., 2004). As P2Y12 activation is not involved in signaling via PLCγ2, it seemed useful to analyse PLCγ2 activation in the presence of the novel thienopyridines to examine specificity of the agents for P2Y12.

Taken together, understanding the mechanism of activation of intracellular signals in platelets such as VASP, PLCγ2, Akt and cPLA2 following P2Y12 engagement, is important to determine the effects of antiplatelet drugs such as novel thienopyridines at the protein level. This study investigates the effect of thienopyridine treatment on downstream signalling events involving VASP, PLCγ2, PLA2 and Akt, with the aim of elucidating the target pathways of the treatment. This will expand on the work of chapter 3 which showed that thienopyridines inhibit end-point platelet activation and aggregation.

4.2 Materials and Methods

4.2.1 Sample preparation

Samples were prepared as described in chapter 2 (section 2.3 & 2.4). Both PRP and washed platelets were used in this section of the study. Briefly, PRP samples were obtained by centrifugation of whole citrated blood for 180 *g* for 15min. The PRP was aspirated from the top of the sample and resuspended in Tyrode's buffer (see section 2.3 For recipe). Washed platelets were obtained by isolation of the PRP as described, followed by addition of 7 nM Prostaglandin E1 (PGE_1) to maintain platelet inactivity during a further centrifugation step at 1400 *g* for 10 min. The platelet pellet was then resuspended in Tyrode's buffer containing 7nM PGE_1.

4.2.2 Platelet Aggregation

Platelet aggregation was assessed using light-transmission aggregometry, as described in section 2.7.

4.2.3 Flow Cytometry Assessment of Ca^{2+} Flux

Intracellular Ca^{2+} spiking following stimulation with platelet agonists was assessed using flow cytometry as described in section 2.8.

4.2.4 Western blotting

Western blotting for phosphorylated VASP (pVASP), total VASP (VASP), phosphorylated Akt (pAKT), total Akt, phosphorylated PLCγ2 (pPLCγ2) and phosphorylated cPLA2 was performed using methods described in section 2.11. Initially PRP samples that had been

used in the aggregation experiments were used for the western blotting. These samples had undergone 5 minutes ADP-stimulated aggregations in the Aggregometer prior to obtain the platelet lysate by addition of RIPA buffer. Further experiments involved washed platelet preparations rather than PRP. However, there were concerns that signalling events had already occurred during the 5 minutes aggregation step and so later experiments involved protein extraction immediately following addition of ADP, rather than following a 5 minutes incubation to ensure that platelet signaling events were not missed.

Briefly, following addition of the 10 µM ADP to the sample (PRP or washed platelet preparation), the sample was immediately centrifuged at 1500 *g* for 5 minute. A 200 µl volume of RIPA buffer was added to the sample containing a 1:1000 dilution of both phosphatase and protease inhibitors to protect platelet proteins from denaturation. The samples were vortexed and iced for 30 min. Vortexing was performed every 5 min until the end of the 30 min duration. Subsequently, mixtures were sonicated for 3 minutes to ensure the breakdown of platelet membranes; and were then frozen at -20°C until further analysis.

Protein assays were performed prior to western blotting using the BCA Protein Assay to quantify the proteins in the platelet lysates prior to Western blotting to normalize lane loading (see section 2.11.1).

For specific protein detection, the following primary antibodies were used: anti-pVASP, anti-pAkt, anti-pPLCγ2 and anti-pPLA2.

4.3 Results

4.3.1 Effect of platelet agonists, and thienopyridines on Ca^{2+} flux.

The initial aim of this part of the work was to establish a robust method for measuring Ca^{2+} flux following platelet activation. Changes in intracellular Ca^{2+} concentration are rapid, often within nanoseconds, thereby posing a challenge in analysis of such responses. To date, Ca^{2+} fluxes have been monitored by confocal microscopy, plate-based assays and flow cytometry, but there are inevitably gaps in the recording of the data, due to the addition of compounds. This comes with significant loss of detail of a rapid Ca^{2+} response. The new generation of flow cytometers allows the addition of platelet agonists with continuous monitoring, providing a method for dynamic Ca^{2+} measurements.

Washed platelets were used for this part of the study and were loaded with Fluo-4-AM before treatment (or not) with thienopyridine derivatives. The samples were then loaded onto the flow cytometer and acquisition was started to establish a baseline fluorescence reading. The lack of necessity for a vacuum system with the Accuri C6 system meant that data could be acquired for 2 minutes followed by immediate addition of the platelet agonist with continual monitoring of the sample. Platelets were gated using FSC/SSC and a baseline fluorescence in FL1-A was established (Figure 4.1A&B). Over the course of 5 minutes, there was no change in baseline fluorescence. It was then important to check whether disturbing the sample by addition of a pipette tip during acquisition would have an effect on the baseline readings. A washed platelet sample was

added to the system and the sample was mixed at 2 minutes into the data acquisition. As seen in Figure 4.1C, there was no effect on fluorescence over the 5 minute period.

Platelet agonists were used to test Ca^{2+} influx using this method. A spike in fluorescence was seen following addition of 1 µM Thrombin and 10 µM ADP respectively at 2 minutes into data acquisition (Figure 4D-F), evidencing that this assay is sensitive and rapid enough to detect Ca^{2+} flux. As expected, the Ca^{2+} flux induced by ADP at 10µM (a weak agonist) was not as significant as that induced by Thrombin at 1µM (a strong agonist), at either concentration.

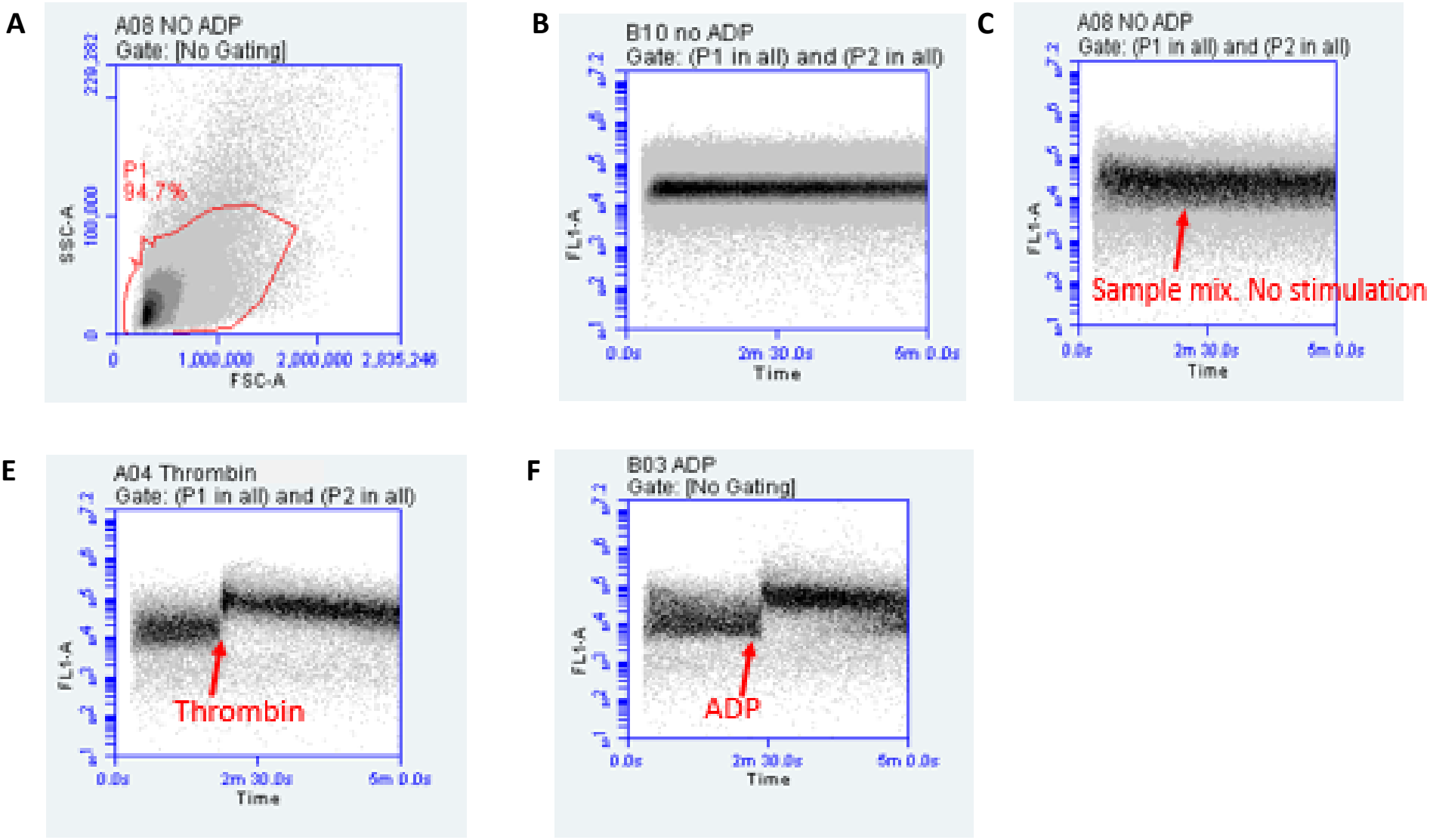


Figure 4.1 Comparative cytograms of Fluo-4 fluorescence versus time, following addition (or not) of Thrombin or ADP at 2 minutes into a 5 minute sample acquisition. (A) gating of the washed platelets in the sample by FSC/SSC. (B) baseline fluorescence of resting platelets (C) effect of mixing the sample intra-analysis, (D) addition of 10µM Thrombin (E) addition of 1µM ADP.

Ca^{2+} flux was assessed in ADP-stimulated washed platelet samples following treatment with theinopyridine compounds. Treatment with cangrelor (Fig 4.2A) abrogated the Ca^{2+} response seen following ADP stimulation (Fig 4.1A). A slight increase in fluorescence was observed following DJ0197 (Fig 4.2B) treatment but this reduced compared ADP stimulation only (Fig 4.1F). DJ0199 appeared as effective as cangrelor in inhibiting ADP-stimulated Ca^{2+} flux (Fig 4.2C). However, Ca^{2+} signaling was still functional following treatment with DJ0021 (Fig 4.2D).

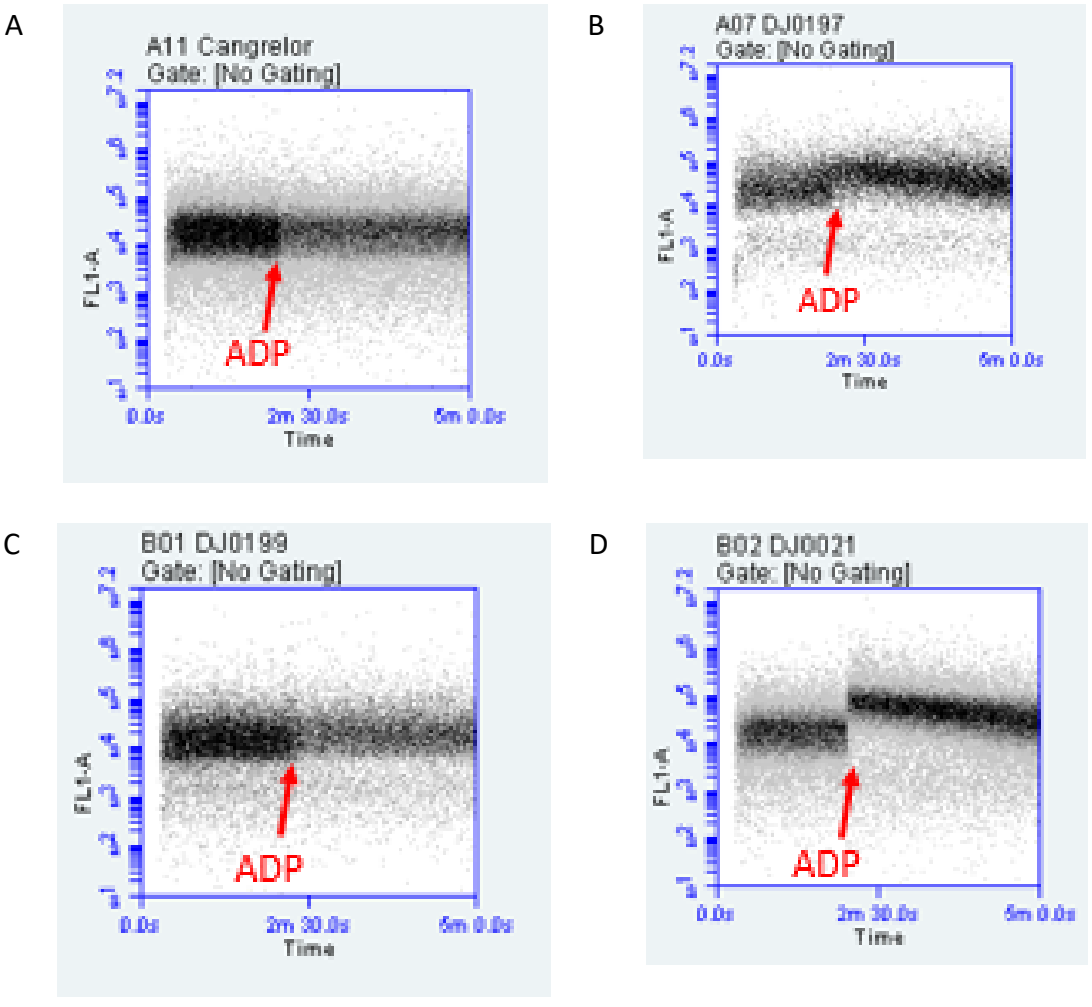


Figure 4.2 Comparative cytograms of Fluo-4 fluorescence versus time, following treatment with (A) Cangrelor (B) DJ0197, (C) DJ0199 (D) DJ0021 prior to addition of 10µM ADP at 2 minutes into a 5 minute sample acquisition.

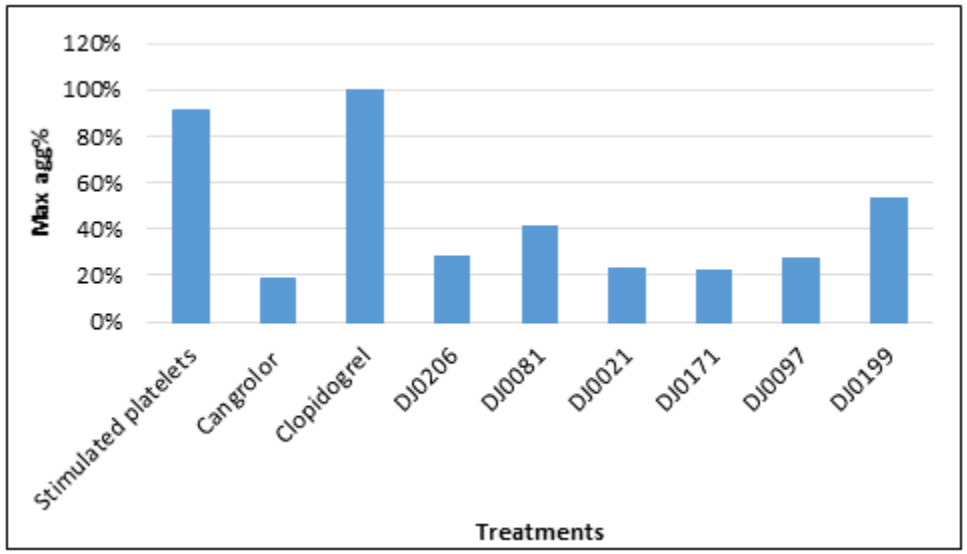
4.3.2 PRP sample aggregation followed by western blotting for pVASP

Initially VASP phosphorylation status was studied in PRP samples following treatment with the thienopyridine derivatives. The use of PRP for these assays mimicked the work carried out in chapter 3 where it was demonstrated that these drugs have the capacity to inhibit both platelet activation and aggregation. PRP samples were pre-treated as described with thienopyridines in section 2.5, simulated with ADP and then allowed to aggregate in the aggregometry chamber for 5 minutes before samples were lysed with RIPA buffer to produce protein extracts in which the protein could be quantified, and the pVASP be analysed by western blot. Figure 4.3 shows the effects of these thienopyridine derivatives on the aggregation (A), and the levels of pVASP (B) in the corresponding samples. The fold difference in pVASP expression compared with control was calculated using densitometry, where pVASP bands were normalized against their relative β-actin density and then compared with the band intensity of the resting (unstimulated) platelet sample. As demonstrated previously, all six novel thienopyridines inhibited the aggregation in response to ADP to a greater degree than clopidogrel, and to an almost comparable level as the reversible $P2Y_{12}$ inhibitor, Cangrelor (Fig. 4.3A). However, analysis of pVASP following aggregation revealed no difference between pVASP levels in resting platelet samples and activated platelets (stimulated with ADP) despite a seemingly increased expression of pVASP following thienopyridine treatment. Another notable anomaly is the lack of effect of Cangrelor on pVASP levels despite the strong inhibitory effect on aggregation.

4.3.3 Use of washed platelet preparations for aggregation followed by Western blotting for pVASP

It was proposed that the use of washed platelet preparations may be more appropriate than PRP for western blot analysis to prevent interference from plasma proteins. The experiments were therefore repeated using washed platelet preparations, pre-treated with the compounds and stimulated with ADP for 5 minutes. Figure 4.4 shows the effects of these thienopyridine derivatives on the aggregation (A), and the levels of pVASP (B) in the corresponding washed platelet samples. Stimulation of the samples with ADP resulted in a reduced level of pVASP (VASP dephosphorylation) in comparison with pVASP levels in resting platelets, verifying that the western blotting method for analysis of pVASP is valid. Although high levels of pVASP were observed in the sample pre-treated with Cangrelor, samples that were pre-treated with other thienopyridine derivatives did not show consistently high levels of pVASP, suggesting that they had not inhibited platelet activation, despite the inhibition shown by aggregometry.

A



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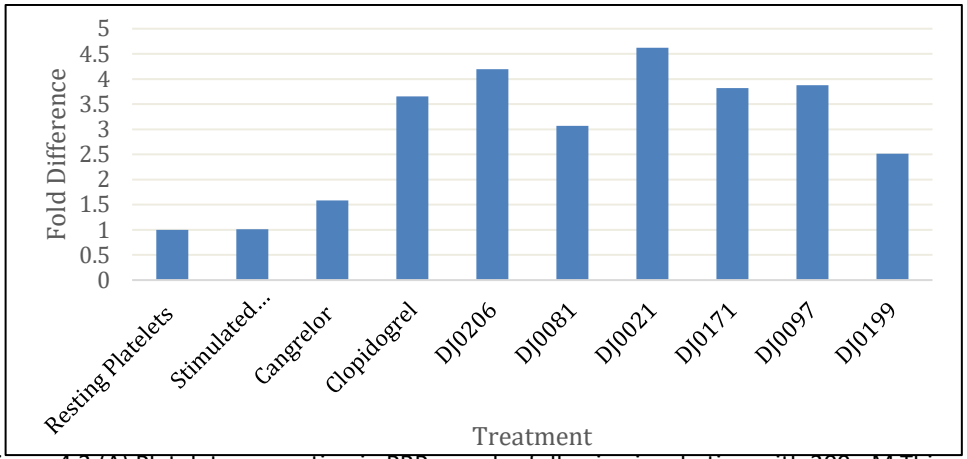

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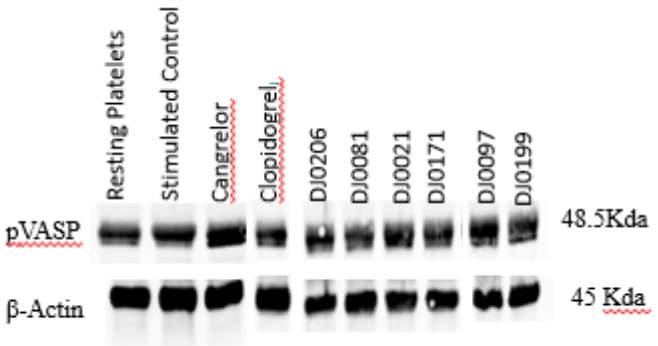
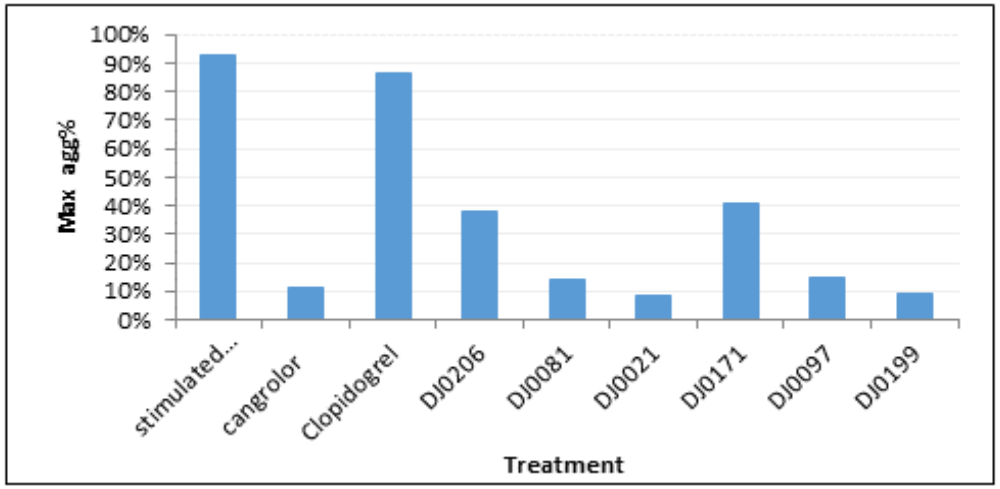


Figure 4.3 (A) Platelet aggregation in PRP samples following incubation with 300 µM Thienopyridine treatment for 30 minutes at 37 °C and stimulated with 10 µM ADP. (B) Fold difference of the intensity of pVASP from the resting control by western blot. (C) Western Blot analysis of pVASP from aggregated samples and lysis by RIPA buffer. Beta actin was used as the loading control n=1.

A



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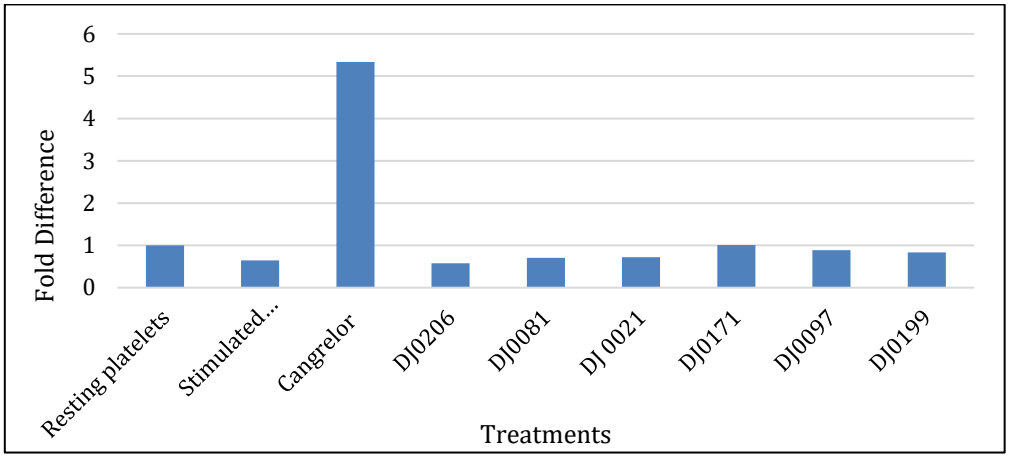

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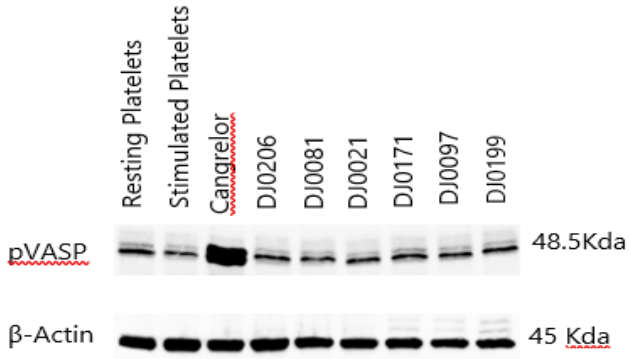


Figure 4.4 (A) Platelet aggregation in washed platelet preparations following incubation with 300 µM thienopyridine treatment for 30 minutes at 37°C and stimulated with ADP. (B) Fold difference of the intensity of pVASP from the resting control. (C) Western Blot analysis of pVASP from aggregated samples and lysis by RIPA buffer. Beta actin was used as the loading control n=1.

4.3.4 Immediate lysis of washed platelet preparations.

Although pVASP levels were maintained in cangrelor-treated samples following stimulation with ADP and a 5 minute aggregation, there was concern that early signalling events may be missed. Therefore the next experiments involved pre-treating samples with thienopyridine derivatives prior to stimulation with ADP and immediate lysis using RIPA buffer.

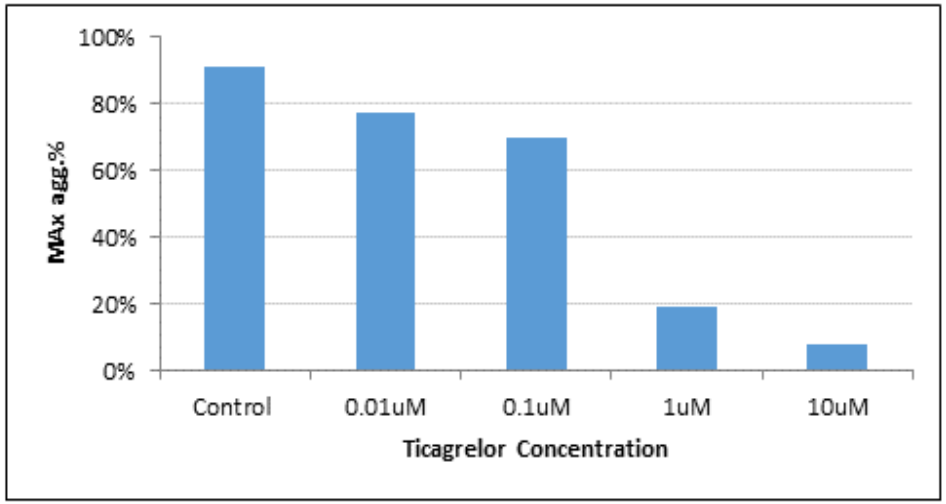
Ticagrelor is a reversible inhibitor of P2Y12, currently prescribed for patients following a myocardial infarction who are at risk of further events. Pre-clinical and clinical data have demonstrated that inhibitory action of this drug on platelet aggregation also platelet signalling. As cangrelor is currently not in clinical use within the UK, and data surrounding its action on G-protein mediated signaling events have not been fully identified, ticagrelor was used in the following experiments to confirm that the methods for platelet lysate extractions and western blotting were valid.

Figure 4.5 illustrates the dose dependent inhibitory effect of ticagrelor on platelet aggregation (A). In line with these findings, the pVASP level was found to be elevated in a dose dependent manner (B). These data confirm that pVASP can be accurately analysed in washed platelet preparations using this method. The de-phosphorylation of VASP following treatment with the six novel thienopyridines in the previous experiment may be due to inhibition via a different platelet signalling pathway. It is important to explore other G-protein mediated events in these treated samples to fully understand the effects of these compounds.

Although pVASP levels were maintained in ticagrelor-treated samples following stimulation with ADP and a 5 minutes aggregation, there was concern that early signalling events may be missed. This was also supported by the data from the Ca^{2+} flux experiments that showed increased intracellular Ca^{2+} levels within seconds following stimulation with ADP (section 4.3.1). Therefore the next experiments involved pre-treating samples with thienopyridine derivatives prior to stimulation with ADP and immediate lysis using RIPA buffer.

In order to limit activation of platelets during sample preparation, PGE_1 was added to the samples during the preparation of washed platelets. Samples were then incubated with thienopyridine derivatives and then stimulated with ADP before immediate lysis with RIPA buffer. Figure 4.6 shows that pVASP levels in samples pre-treated with DJ0206, DJ0081 and DJ0021 did not appear different from those in the activated platelets, however, higher pVASP levels were observed in those samples pre-treated with DJ0171, DJ0097 and DJ0199 indicating lower platelet activation in these samples. This experiment was repeated to check the reliability of this method and the results showed it is a reliable experiment. Fig 4.6C shows the average pVASP fold difference in band intensity of six repeated experiments that approved these three treatments (DJ0171, DJ0097 and DJ0199) resulted in a lower platelet activation compared to other compounds.

A



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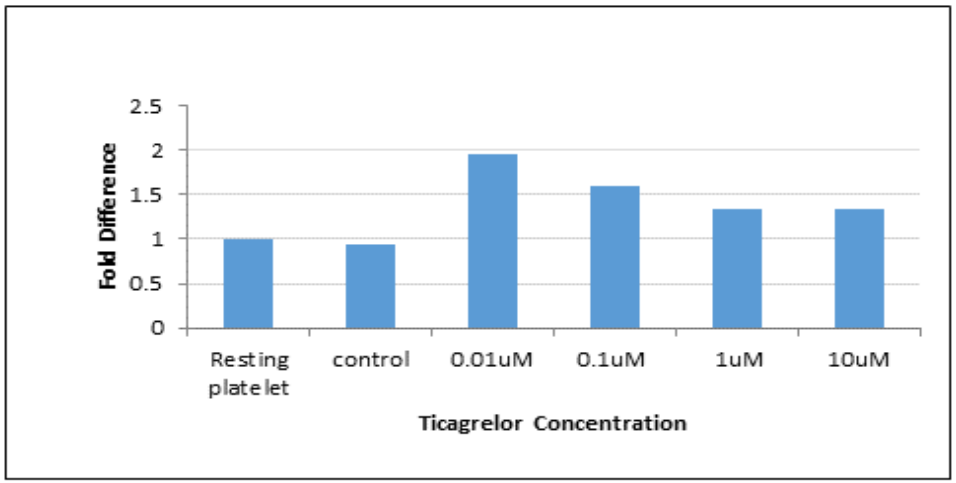

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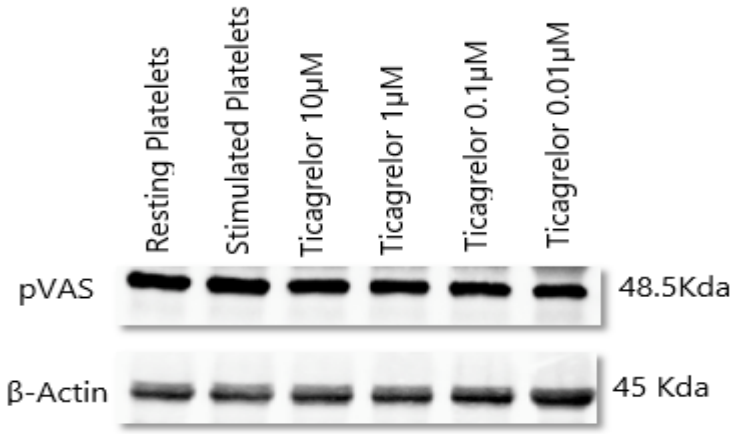
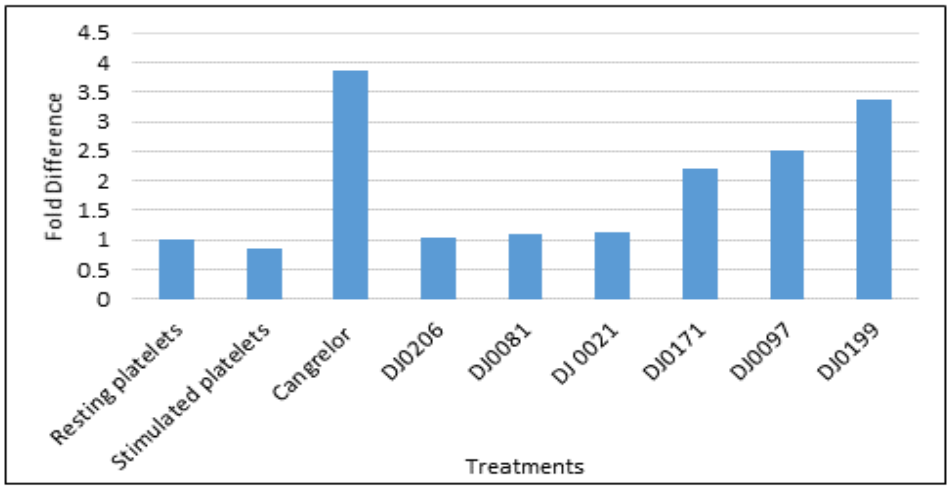


Figure 4.5 (A) Platelets aggregation of washed platelet samples following incubation with different concentrations of Ticagrelor for 5 minutes at 37°C and stimulation with 10 μM ADP. (B) Fold difference of the intensity of pVASP from the resting control. (C) Western Blot analysis of pVASP from aggregated samples and lysis by RIPA buffer. Beta actin was used as the loading control n=1.

A



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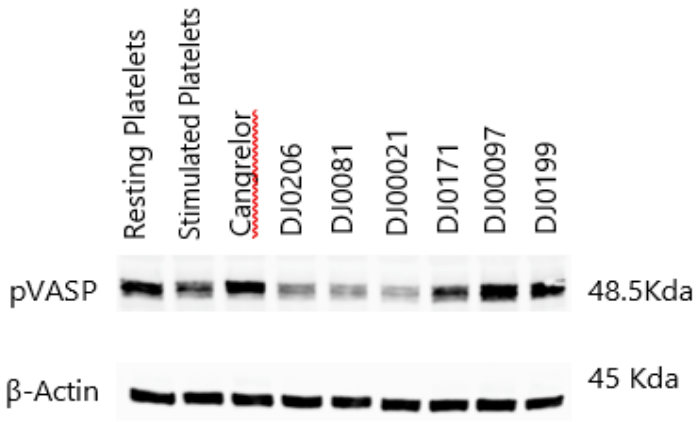

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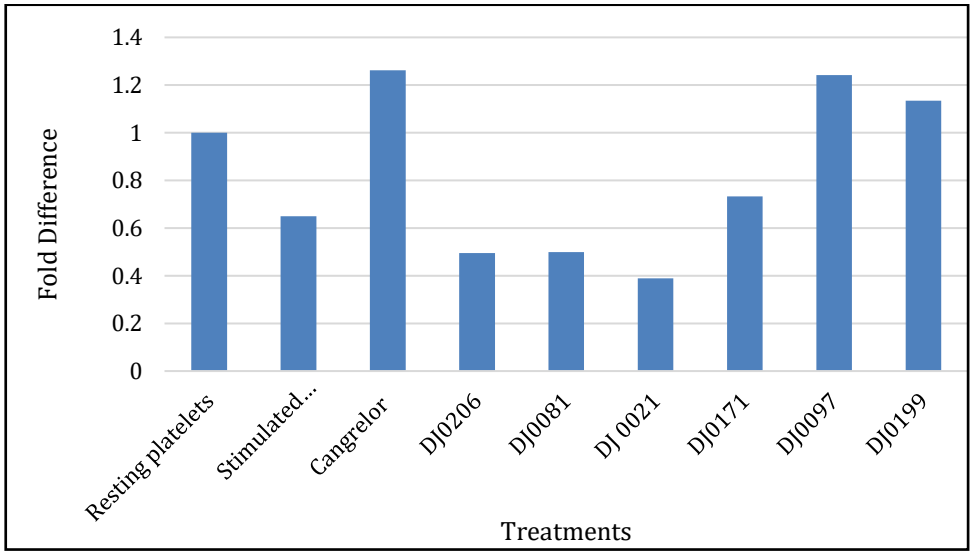


Figure 4.6 (A) Fold difference of the intensity of pVASP from the resting control of washed platelets incubated with 300 µM Thienopyryidine treatments and stimulated with ADP. (B) Western Blot analysis of pVASP from aggregated samples and lysis by RIPA buffer. Beta actin was used as the loading control. (C) Average fold difference of pVASP from the resting control of washed platelets incubated with Thienopyridine treatments stimulated with ADP n=6

4.3.5 Western blot analysis of phosphorylated Akt, PLCy2 and PLA2.

The effects of these thienopyridine treatments on other G-protein mediated signalling events were also assessed by western blotting. Downstream signalling of P2Y12 includes phosphorylation of cPLA2, AKT and PLC (Puri, 1998; Mangin et al., 2003; Kim et al., 2004). The isoform of PLC activated following stimulation with particular agonists is unclear, with suggestions that collagen mediated activation activates PLCy2, while ADP stimulates PLCβ (Stalker et al., 2012). However, there are contradictions in the literature surrounding this. PLC hyrdrolizes PIP_2, producing the second messenger inositol-1,4,5-trisphosphate (IP_3) which is needed to raise the cytosolic Ca^{2+} concentration. The theinopyridines were shown to inhibit this Ca^{2+} flux and therefore must have an inhibitory effect on PLC.

Following platelet Gi-coupled P2Y12 receptor stimulation, the subunits of Gi protein (α_{Gi} and βγ) are released. The βγ subunit can suppress protein kinase C but activate the phosphatidylinositol 3-kinase (PI-3K), which is responsible for both Akt and Rap1b regulation by phosphatidylinositol-dependent kinase-1 (PIDK1). The generated Akt, as well as Rap1b, contributes to activation of the platelet fibrinogen receptor, resulting in platelet aggregation (Joo, 2012).

Another event following ADP-mediated activation, is the phosphorylation of $cPLA_2$ due to the mobilised Ca^{2+} from the platelet dense tubular system. This causes the contents of platelet granules to be secreted and the platelet fibrinogen receptor to be activated. Activating $cPLA_2$ also leads to form AA by splitting the fatty acids, particularly phosphatidylethanolamine and phosphatidylcholine, at the sn-2 position. The platelet

PTGS1 (also called cyclooxygenase 1 (COX-1)) enhances the conversion of AA into the cyclic endoperoxide prostaglandins (PGG2 and PGH2), that are transformed into $TBXA_2$ by TBXA synthase, leading up to amplified aggregation of the platelet (Stassen et al., 2004).

To confirm the effects of P2Y12 stimulation and inhibition on $cPLA_2$ phosphorylation, washed platelets pre-treated with ticagrelor (or with untreated control), were stimulated with ADP and the results (Fig. 4.7A) show ticagrelor inhibit platelet aggregation then the samples were immediately lysed using RIPA buffer. As shown in Figures 4.7 B and 4.7C, ADP stimulation resulted in an increase in phosphorylated cPLA2. However, ticagrelor was able to prevent this increase in a dose-dependent manner (Fig. 4.7B and C).

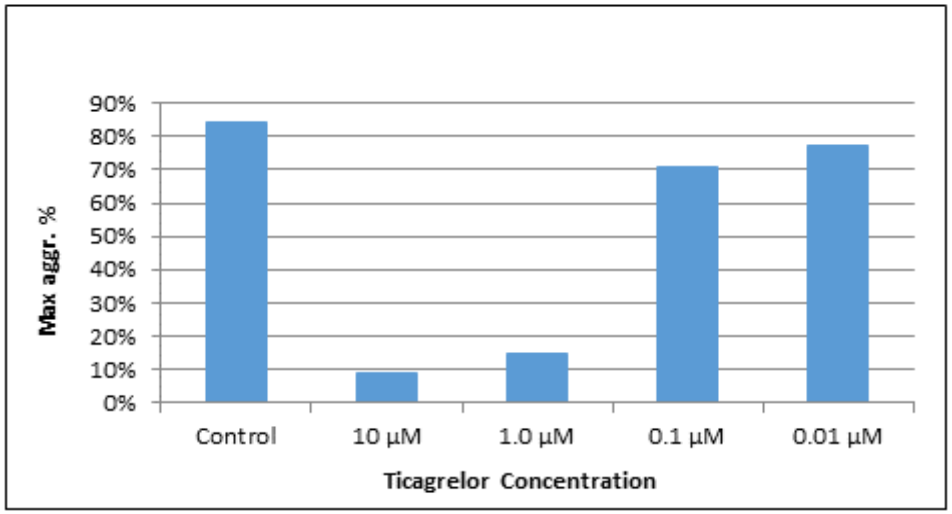
The phosphorylation status of $cPLA_2$, Akt, and PLCγ was assessed in washed platelets pre-treated with thienopyridine derivatives and stimulated with ADP followed by immediate lysis in RIPA buffer. Figure 4.8 shows the expression of pAkt (A&B), p-cPLA2 (C&D) and p-PLC-γ2 (Fig. 4.9A&B) in these treated samples. The first observation, is that stimulation of platelets with ADP resulted in an increase in pAkt (Fig. 4.8A) in comparison with resting platelets, which is confirmation of previous literature. Cangrelor prevented this stimulation, highlighting its inhibitory effect on platelet activation. However, the thienopyridine derivative-treated samples showed elevated levels of pAkt which is in contrast to their inhibitory effects on aggregation.

Following ADP-induced activation, cPLA2 is phosphorylated to p-cPLA2, which in turn releases arachidonic acid (AA) for the biosynthesis of prostaglandins and thromboxanes via the cyclooxygenase system (Gibbins et al., 2004).

Figure 4.8C shows an increase in pcPLA2 following stimulation with ADP, in comparison with resting platelets which demonestrates the effect of ADP on cPLA2. However, cangrelor did not show the inhibitory effect on pcPLA2 despite of cangrelor effect on P2Y12 receptor which is ADP receptor on platelets. This may open a gate of the other effect of ADP on pcPLA2 through different receptors which could be P2Y1. Moreover, thienopyridine treatment did not show obvious effects on pcPLA2 compared to stimulated platelets.

pPLC γ2 (Fig. 4.9A) appeared unaffected by thienopyridine treatment when compared to the resting platelets when they have pre- treated with thienopyridine. However, pPLCγ2 seems to not to be a downstream signal to the ADP receptor as the stimulated platelet not change when or increase in compare to resting platelet.

A



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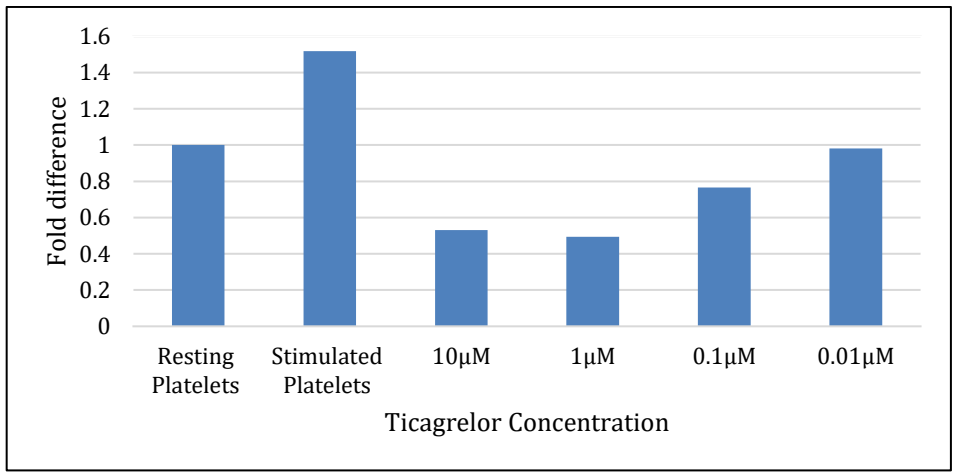

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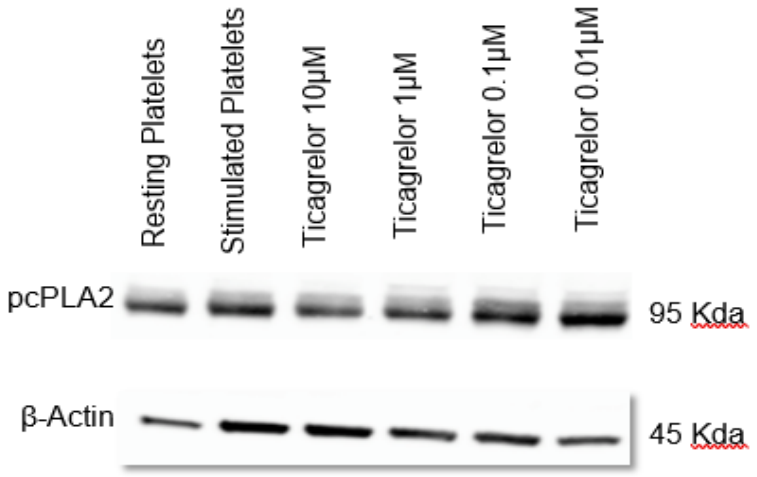


Figure 4.7 (A) Fold difference of the intensity of pcPLA2 from the control. (B) Westren Blot bands of pcPLA2 from aggregated samples and lysis by RIPA buffer where Beta actin used as loaded control (C) Platelet aggregation of PRP samples that incubated with different concentration of Ticagrelor for 5 minutes at 37 ˚C and stimulated with 10 µM ADP n=1.

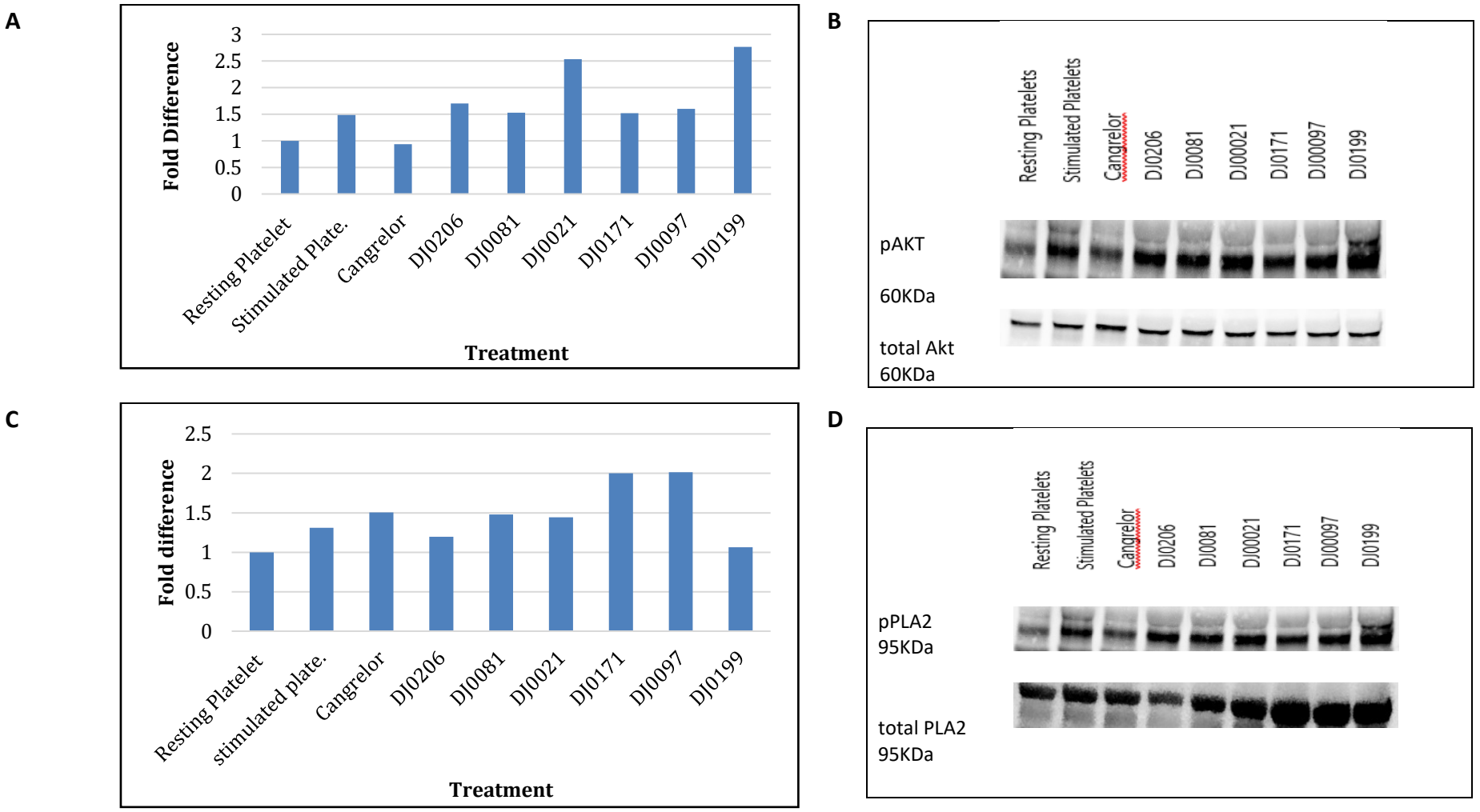
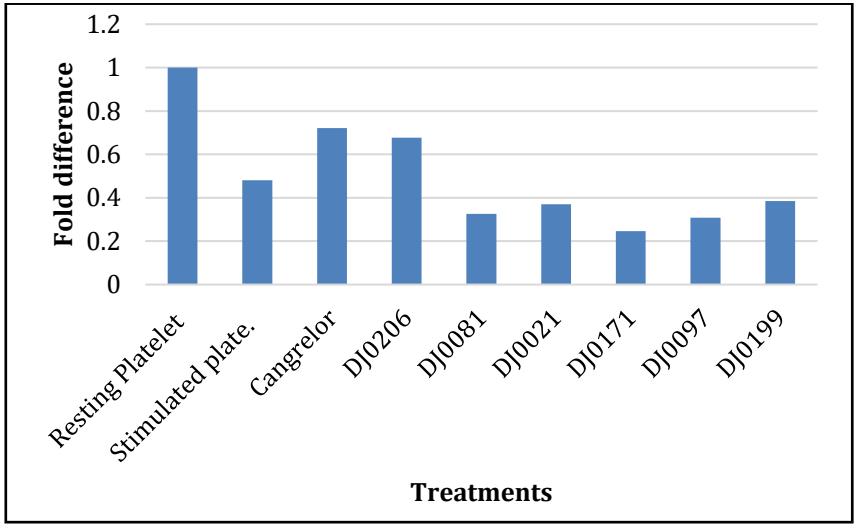


Figure 4.8 (A) Fold difference of the intensity of pAkt from the resting platelets that incubated with 300 µM of Thienopyryidine treatments. (B) Westren Blot bands of pakt from incubated platelets and lysis by RIPA buffer where total Akt used as loaded control. (C) Fold difference of the intensity of pPLA2 from the resting platelets that incubated with 300 µM of Thienopyryidine treatments. (D) Western Blot bands of pPLA2from incubated platelets and lysis by RIPA buffer where total PLA2 used as loaded control n=2.

A



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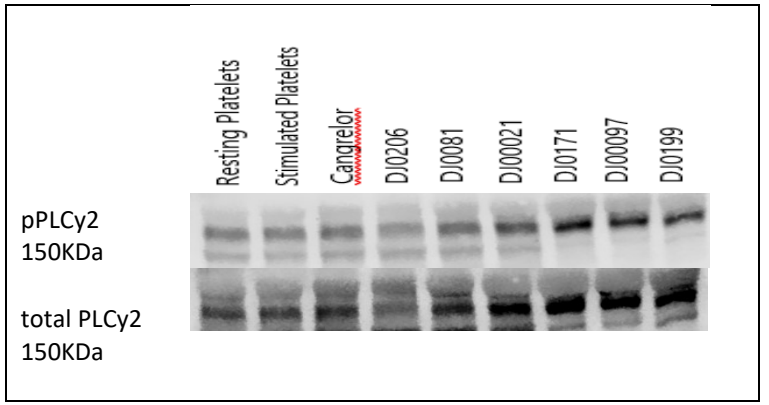


Figure 4.9 (A) Fold difference of the intensity of pPLCy2 from the resting platelets that incubated with 300 µM of Thienopyryidine treatments. (B) Westren Blot bands of pakt from incubated platelets and lysis by RIPA buffer where total PLCy2 used as loaded control n=1.

4.4 Discussion.

Despite ADP being a weak agonist compared to thrombin and collagen, it is important in the recruitment of additional platelets to sites of injury (Dorsam and Kunapuli, 2004). When ADP is added to platelets in-*vitro* it causes TXA2 formation, protein phosphorylation, increased Ca^{2+} influx, shape change and platelet aggregation (Gibbins et al., 2004). As the P2Y12 receptor is important in ADP functions, blocking this receptor by ticagrelor, cangrelor, or clopidogrel is important to control thrombosis in cardiovascular diseases, demonstrated to be clinically effective in the prevention of myocardial infraction, ischemic stroke and vascular death (Nurden et al., 2001; Dorsam and Kunapuli, 2004). Moreover, downstream signalling from the P2Y12 receptor is G_i signalling which leads to a reduction in the level of cAMP, activation of PI3K, Akt and Rap1b and influx potassium channels (Dorsam and Kunapuli, 2004; Shankar et al., 2004).

This chapter of work started by investigating the effect of thienopyridines on Ca^{2+} signalling. The results demonstrated that real-time flow cytometry is an excellent method for allowing assessment of Ca^{2+} following addition of an agonist mid-sample analysis (Fig 4.1). A change in fluorescence was observed following stimulation of washed platelets with ADP and Thrombin. Furthermore, DJ0199 and DJ0097 (as well as cangrelor) were both shown to inhibit the Ca^{2+} response to ADP, while DJ0021 appeared to be relatively ineffective (Fig 4.2). However, despite these clear-cut results, a great deal of optimisation was involved this assay, and the sensitivity to ADP did seem to disappear very quickly following preparation of the samples, meaning that inconsistent results were obtained (data not shown). However, response to thrombin in this assay

was extremely consistent, and the data provide justification for adopting this assay more routinely in platelet signalling research.

Next, phosphorylation of VASP was assessed using Western Blotting, as a marker to evaluate P2Y12 antagonism (Hezard et al., 2005). However, due to variability in the response to clopidogrel between individuals, the study utilised another positive control Cangrelor, as a direct reversible antagonist of P2Y12 (ADP receptor) (Hezard et al., 2005). Studying the signal by western blot required the tested drug to have a clear difference between negative and positive control samples.

The main finding from this study is that specific novel thienopyridines maintain the phosphorylation of VASP when platelets are stimulated with 10µM ADP. This concentration of ADP is supported by the study of Pampuch et al. (2006) who demonstrated the correlation of analysing VASP, LTA and flow cytometry when following cangrelor treatment and stimulating with 10µM ADP (Pampuch et al., 2006). VASP phosphorylation status has been shown to be an extremely reliable marker of platelet activation (Mallouk et al., 2018); (Hezard et al., 2005). Indeed, western blotting analysis has been considered as a robust qualitative method (Dorsam and Kunapuli, 2004; Hezard et al., 2005; Zhang et al., 2017).

This chapter intially investigated pVASP in the same samples that had been used for aggregation analysis (Fig. 4.3). The samples were first tested by LTA and then lysed for western blot analysis. However, the results from western blots were not consistent with the LTA results. The proposed reason could be due to interference of other proteins in

the PRP samples in the test and the second reason from the delay in lysing platelets after aggregation assessment.

To counteract these issues, platelets were incubated with thienopyridine, activated with ADP and lysed immediately. This technique proved successful in the study of VASP (Fig. 4.6), Akt (Fig. 4.7A&B) and PLA2 (Fig. 4.7B&C). In contrast the study of Hezard et al., used PRP samples and incubated platelets with ADP or the PGE1 inhibitor for 5 minutes (Hezard et al., 2005). This showed the difference in platelet VASP phosphorylation by PGE1 or dephosphorylation by ADP.

This chapter also investigated the effect of other signaling events that may be involved in downstream signalling of the P2Y12 receptor, and could be affected by blocking P2Y12 with novel thienopyridines. One such downstream signalling molecule is Akt, which is important in platelet activation (Kim et al., 2004). The results of this study showed thienopyridines did not inhibit Akt phosphorylation once platelets were induced by ADP, whereas cangrelor (positive control) did (Fig. 4.7A&B). The data demonstrating activation of Akt by ADP is supported by the study of Kim (2004) which stimulated platelets from mice deficient in P2Y1 with ADP. Their results showed activation of Akt signaling, illustrating the specificity of P2Y12 in Akt activation. In addition, Akt activation was inhibited in platelets from these mice which were treated with clopidogrel and induced by ADP. Furthermore, this demonstrates the mechanism of activating Akt by the G_i and P2Y12 receptor complex (Kim et al., 2004; Carrié et al., 2009).

Although Akt has been shown to be activated only by P2Y12, ERK has been shown to be activated by the presence of either P2Y1 or P2Y12. Since ERK may signal through the P2Y1 receptor which is not blocked by clopidogrel or cangrelor, it was not investigated in the present study (Garcia et al., 2007).

With regards to cPLA2, the results show no effect of thienopyridine or cangrelor, despite ADP leading to increased expression in stimulated platelets compared to resting platelets (Fig. 4.7B&C). Strangely, the alternative P2Y12 inhibitor ticagrelor was shown to inhibit cPLA2 in a dose dependent manner. Literature on the involvement of cPLA2 in signalling downstream of P2Y12 is weak (Garcia et al., 2007), but De Cristofaro et al (1989), reported that cPLA2 was indirectly activated following ADP stimulation due to the Na^+ /H^+-mediated increase in cystolic pH, increased Ca^{2+} levels (De Cristofaro et al., 1989). Moreover, it has been reported that Ethyllisorpopylamilorode, a disrupter of Na^+ /H^+ exchange decreases the cytosolic pH which blocks ADP induction of cPLA2 (Puri, 1998). cPLA2's involvement in signalling following thrombin stimulation is much more established (Puri, 1998). As the results from the present study did not provide consistent results between cangrelor and ticagrelor, the role of cPLA2 in P2Y12 signalling remains to be determined.

With regards to the PLCγ2 results from this study, ADP stimulated samples did not appear to show activation of PLCγ2, and neither cangrelor nor the novel thienopyridines had any affect (Fig. 4.9). As mentioned earlier, the literature reports that collagen is the predominant activator of PLCγ2 via GPVI-mediated association of the FcRy chain (Watson et al., 2001). PLCγ2 works downstream from the $\alpha_2\beta_1$ and GPVI receptors. A

study using amarogentin, an active ingredient of the Gentiania Lutea plant, demonstrated inhibition of platelet activation induced by collagen but not by thrombin or archidonic acid (Yen et al., 2014). Amarogentin was found to inhibit PLCγ2 in collagen-stimulated platelets via PLCγ2-PKC-P47 pathway in conjunction with the MAPK signalling cascade (Yen et al., 2014). The work on amarogentin supports the results from this chapter. Collagen-induced platelet activation leads to release of secondary agonists such as ADP and TXA2 which then appear to be involved in PLCβ activation, another of the PLC isoforms (Mangin et al., 2003). This activation of PLCβ occurs through $G_{\alpha q}$ protein coupled receptor (Mangin et al., 2003). Moreover, platelet aggregation has been reported to be normal in PLCγ2 deficient mice when platelets are stimulated with ADP or thrombin, whereas the aggregation is abolished when stimulated with collagen. ADP receptor antagonists in these mice lead to inhibition of shape change when platelet activated by ADP (Wang et al., 2000).

In conclusion, this chapter demonstrated that the novel thienopyridines DJ0171, DJ0097 and DJ0199 were able to maintain VASP phosphorylation in the presence of ADP, with pVASP levels significantly higher in treated platelets than in ADP-simulated platelets. However, effects on pAkt, pcPLA2 and pPLCγ2 were inconsistent. The real-time flow cytometry assay was shown to be a robust method for analysis of Ca^{2+} flux in platelets. Further work would act to clarify the involvement of PLCβ in P2Y12 signalling, and the effects of these novel compounds on this protein kinase.

Chapter 5: Pilot Study – The Effect of Novel Thienopyridines on Endothelial Cells

Chapter 5: Pilot Study – The Effect of Novel Thienopyridines on Endothelial Cells.

5.1 Introduction

Endothelial cells play a vital role in haemostasis, with both antithrombotic and prothrombotic properties that work in a balanced system to maintain blood fluidity and prevent thrombus formation (Wu and Thiagarajan, 1996). Cell-cell communication between endothelial cells with platelets has only recently begun to receive systematic study, but it is understood that they communicate in a paracrine fashion (over relatively-long distances), via transient local mechanisms and through receptor-mediated cell-cell interactions (Siegel-Axel and Gawaz, 2007).

Under normal conditions, microenvironmental nitric oxide (NO) inhibits the expression of cellular adhesion molecules on the endothelium, keeping the cells in a 'resting state' NO also contributes to maintaining a platelet resting state. Indeed, in the presence of pro-inflammatory cytokines, a much higher concentration of NO is required to prevent expression of adhesion molecules and recruitment of leukocytes to the local area(Liao, 1998). Inhibition of endogenous endothelial NO production by NG-monomethyl-l-arginine has been shown to induce the adhesion molecule VCAM-1 expression in cultured endothelial cells (De Caterina et al., 1995; Khan et al., 1996).

In addition to its presence in platelet alpha-granules, CD62P (P-selectin) is expressed on the endothelial cell surface upon activation following release from Weibel-Palade bodies located in the cytosol (Mark I. Furman et al., 1998; Kuckleburg et al., 2011; Ley et al.,

2011). The CD62P acts as a cell adhesion molecule, binding to PSGL1 which is found on various hematopoietic cells such as neutrophils, eosinophils, lymphocytes, and monocytes. CD62P is therefore vital in the recruitment of leukocytes to the site of vessel injury, and this works in conjugation with platelet-expressed CD62P which, as described in chapter 3, allows for the formation of platelet-leukocyte aggregates (Huo et al., 2003; Nofer et al., 2010; Verhamme and Hoylaerts, 2006). Levels of CD62P (along with another selectin CD62E) have been found to be elevated in patients with Acute Myocardial Infarction (AMI) (Sakurai et al., 1997); providing justification for investigating changes in expression of this molecule in response to P2Y12 inhibition.

CD62E (E-Selectin) is expressed only on endothelial cells following stimulation with pro-inflammatory cytokines. It is arguably the most specific, inducible endothelial cell-surface molecule involved in the adhesion of neutrophils, monocytes, and T cell subsets to the local area. Its expression has been shown to be upregulated in cases of ACS and atherosclerosis. Interestingly, clopidogrel has been shown to retard the development of atherosclerotic plaques in mouse models and to significantly reduce both CD62P and CD62E expression when compared to non clopidogrel treated mice (Heim et al., 2016).

Intercellular adhesion molecule-1 (ICAM-1; CD54) is a 90 kDa member of the immunoglobulin (Ig) superfamily and is critical for the arrest and migration of leukocytes out of blood vessels and into the tissues. ICAM-1 is upregulated by pro-inflammatory cytokines on the surface of endothelial cells (Lawson and Wolf, 2009) and can be shed into the extracellular milieu as soluble ICAM-1) (sICAM). sICAM-1 can be detected in various body fluids, with elevated levels being observed in patients with atherosclerosis,

heart failure, coronary artery disease and transplant vasculopathy (Macías et al., 2003; Lawson and Wolf, 2009), indicating its association with endothelial dysfuction/over-activation. It is proposed that ICAM-1 directly contributes to inflammatory responses within the blood vessel wall by itself increasing endothelial cell activation in the local area and augmenting atherosclerotic plaque formation and can upregulate its own expression via a positive-feedback loop. ICAM-1 has been shown to be over-expressed in human atherosclerotic lesions. There is therefore justification for looking at whether P2Y12 inhibitors have the ability to downregulate ICAM-1 expression on endothelial cells (Lopes-Virella and Virella, 1992).

Vascular Cell Adhesion Molecule-1 (VCAM-1) is another cell adhesion molecule involved in the recruitment of leukocytes to the site of injured vessel. Unlike ICAM-1 that is expressed at low levels on non-activated endothelial cells, VCAM-1 is only expressed by endothelial cells upon activation (Granger and Senchenkova, 2010). In addition to sICAM-1, sVCAM-1 shed from the activated endothelial cells has been found to be elevated in patients with ACS when compared with healthy control patients, and sVCAM-1 can strongly predict an increased risk for subsequent cardiovascular events in such patients (Postadzhiyan et al., 2008). Again, there is justification for investigating the effects of P2Y12 inhibitors on the expression levels of this protein.

Many of the platelet agonists that have been discussed and utilised throughout this thesis have important roles at the endothelial cell level. ADP has a significant role in inducing the migration of human endothelial cells via the interaction with endothelial-P2Y1 receptor expression. This mechanism is one of several that contribute to angiogenesis during vascular repair (Shen and DiCorleto, 2008). Other P2Y receptor

family members are known to be expressed by endothelial cells. Shen et al. (2008) reported very high levels of P2Y1 on endothelial cells, with lower levels of P2Y12. This same study also demonstrated a MAPK downstream signalling cascade involving phosphorylation of Erk1/2, JNK, and p38 following ADP stimulation. Hess et al. (2009) also showed phosphorylation of eNOS following ADP treatment, thereby indirectly demonstrating ADP-mediated Ca^{2+} flux in these cells. Kaczmarek et al. (2005), demonstrated that ATP is also capable of inducing Ca^{2+} flux in this cell type. ATP has been shown to enhance vasodilation via the P2X4, while in vascular smooth muscle cells (VSMC), ATP appears to cause vasocontriction via P2X1. P2Y2 and P2Y11 have also been shown to be present at the mRNA level in endothelial cells (Shen and DiCorleto, 2008). Upon ADP stimulation, endothelial cells have been shown to release PGI2 and NO which act to inhibit platelet activation (Shen and DiCorleto, 2008). Thereby suggesting an antithrombotic role. This is in contrast to the effects of ADP on platelets themselves, but suggests a mechanism by which the endothelial cells limit the thrombosis to the local area.

This chapter describes data obtained from pilot experiments, designed to assess the effects of P2Y12 inhibitors on expression of key adhesion molecules by endothelial cells. This was firstly to determine whether the endothelial dysfunction reported in patients with concomitant platelet hyperactivity is also being addressed by these same medications, and secondly to determine whether the novel thienopyridine compounds had such as effect.

5.2 Methods

5.2.1 Cell culture

Human umbilical vein endothelia cells (HUVEC) line was purchased from Cattag Medsystem (Buckingham, UK). The cells were cultured as described in section 2.9.

5.2.2 Sample preparation for treatment

Between passages 3 to 8, the HUVEC cells (4.6 $x10^4$) were used for thienopyridine treatments. The cells were cultured in 24 well plates over glass cover slips that had been coated with 0.1% gelatin. The cells were allowed to settle in the centre of the coverslips for 30 minutes before addition of M199 medium to avoid growing the cells at the edges of the well outside the cover slip. After two days, the cells were washed twice with phosphate buffer saline (PBS) (Lonza, UK) and incubated with low serum 5% (FBS) M199 overnight. Thereafter, the cells were washed twice ready for thienopyridine or TNF-α treatments. The cells were at approximately 80% confluence on the coverslips prior to treatment.

5.2.3 HUVEC cell treatments.

HUVEC cells were treated with 100ng/ml TNF-α (Promo Cell, UK) for 4 hours with fresh media M199 with low serum FBS to avoid the interference with the treatment effect on cells. A 300 µM concentration of each thienopyridine treatment was applied to the cells for 30 minutes at 5% CO2 at 37°C. Ticagrelor was used as the comparison. At the end of the treatment period, 10µM ADP was added to the samples including the untreated

control and incubated for 5 minutes. Cells were washed twice with PBS before fixation and labelling.

5.2.4 Fixation and labelling

As it described in section 2.10, The cells were fixed with 0.5% paraforlmaldehyde for 2.5 minutes then washed with PBS. After that, the cells were permeabilised in 1% triton X-100 for 8 minutes, then blocked using 3% bovine serum albumin (BSA) for 1 hour. The cells then were incubated with the following monoclonal antibodies at a 1:300 dilution: Mouse Anti-human P-selectin, Rabbit anti-human E-selectin, Mouse anti-human ICAM-1 and rabbit anti-human VCAM-1. The samples were incubated overnight in the fridge in a dark environment. Secondary antibodies (used at a 1:400 dilution) were added: Alexa 568 (Rhodamine)-Anti-rabbit IgG added to all samples which was used to detect E-selectin in one sample and VCAM-1 in another. Alexa 647 (Cy5)-Anti-mouse IgG was used to detect P-selectin and ICAM in the different samples. The secondary antibodies were incubated in the dark for 1 hour at room temperature. The cells were then washed twice with PBS before 1 drop mounting media with 4,6 diamidino-2-phenylidole (DAPI) was added to the sample to stain the genetic material in the cells. The coverslips were then mounted on to glass microscopy slides. The samples were kept in the dark until analysis by epifluorescence microscopy (Zeiss imager Z1 microscope) under 10x and 40x oil immersion lens.

5.3 Results

5.3.1 Effect of thienopyridine treatments on ADP-induced HUVEC cell expression of P-selectin and E-selectin

TNFα was used as a positive control in this study due to the well established stimulation of CD62P and CD62E on endothelial cells by this cytokine. The images from the epifluorescence microscopy illustrate the effect of stimulators on HUVEC cells expression of CD62P and CD62E.

High expression of P-selectin (green) and E-selectin (red) on HUVECs was observed following treatment with TNFα, confirming the validity of the method (Fig 5.1A). Treatment with ADP was also shown to induce the expression of both adhesion molecules (Fig 5.1B).

An exciting finding was that treatment with thienopyridines resulted in absence of CD62P expression in ADP-stimulated HUVEC cells (Fig 5.1C and Fig 5.2 D-G). CD62P was also absent in the ticagrelor treated samples. This was not the case for CD62E, which did appear to be expressed in most samples. However, a complete absence of the protein was observed following ticagrelor treatment, with lower intensities also observed DJ0171 and DJ0199, when compared to the other treatments.

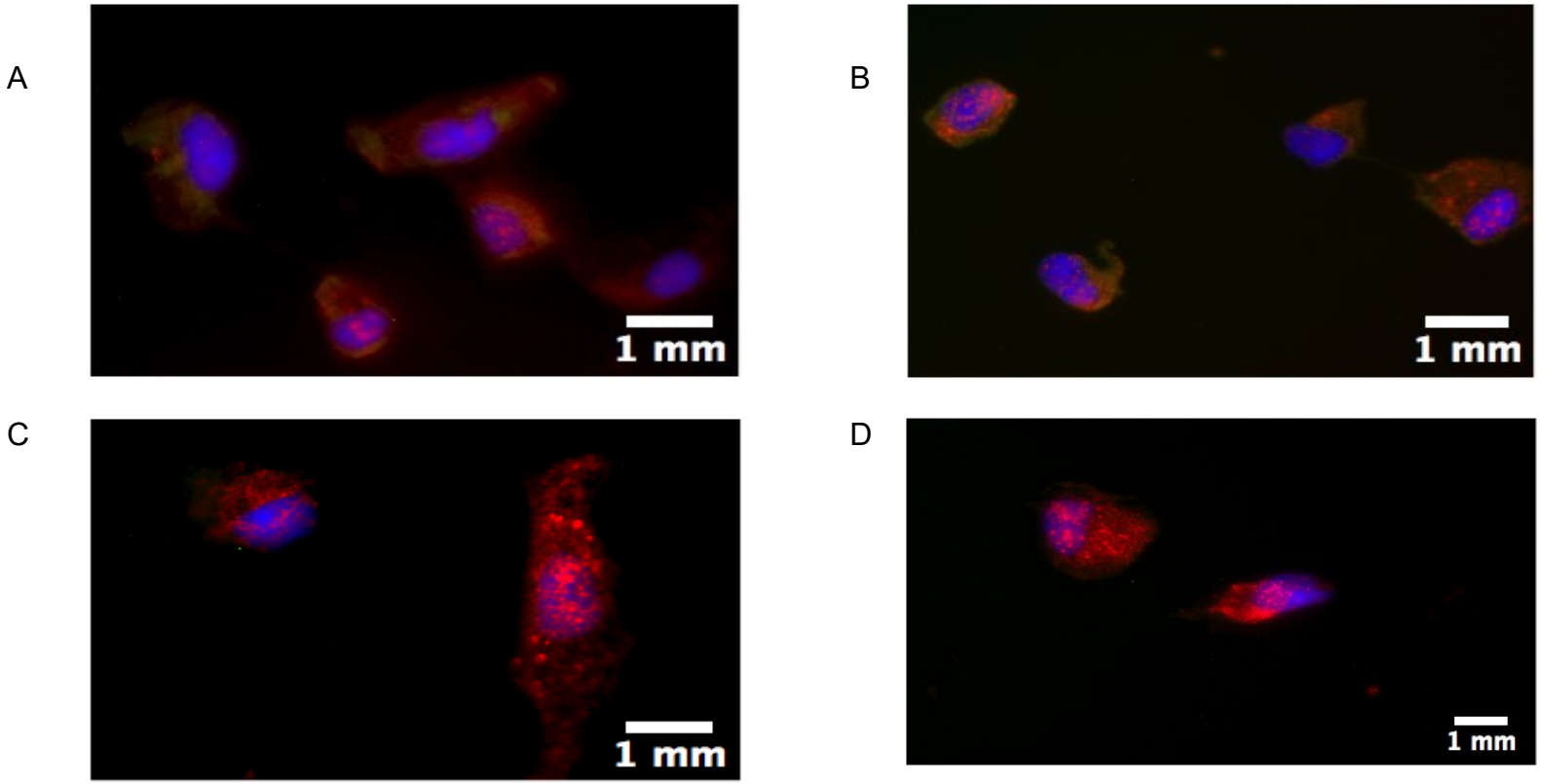


Figure 5.1 Epifluorescence microscopy pictures of P-Selectin (green), E-selectin (red) and DAPI (blue) in HUVEC cells following different treatments. HUVEC cells were incubated with (A) TNF-α for 4 hours as positive control. (B) ADP only for 5 minutes, (C) DJ0206. (D) DJ0081. (E)DJ0021. (F)DJ0171 (G) DJ0097 H) DJ0199 and (I) Ticagrelor. For C-I, the cells were treated with P2Y12 inhibitor for 30 minutes prior to 5 minute ADP stimulation and investigated on 40x oil emmersion.

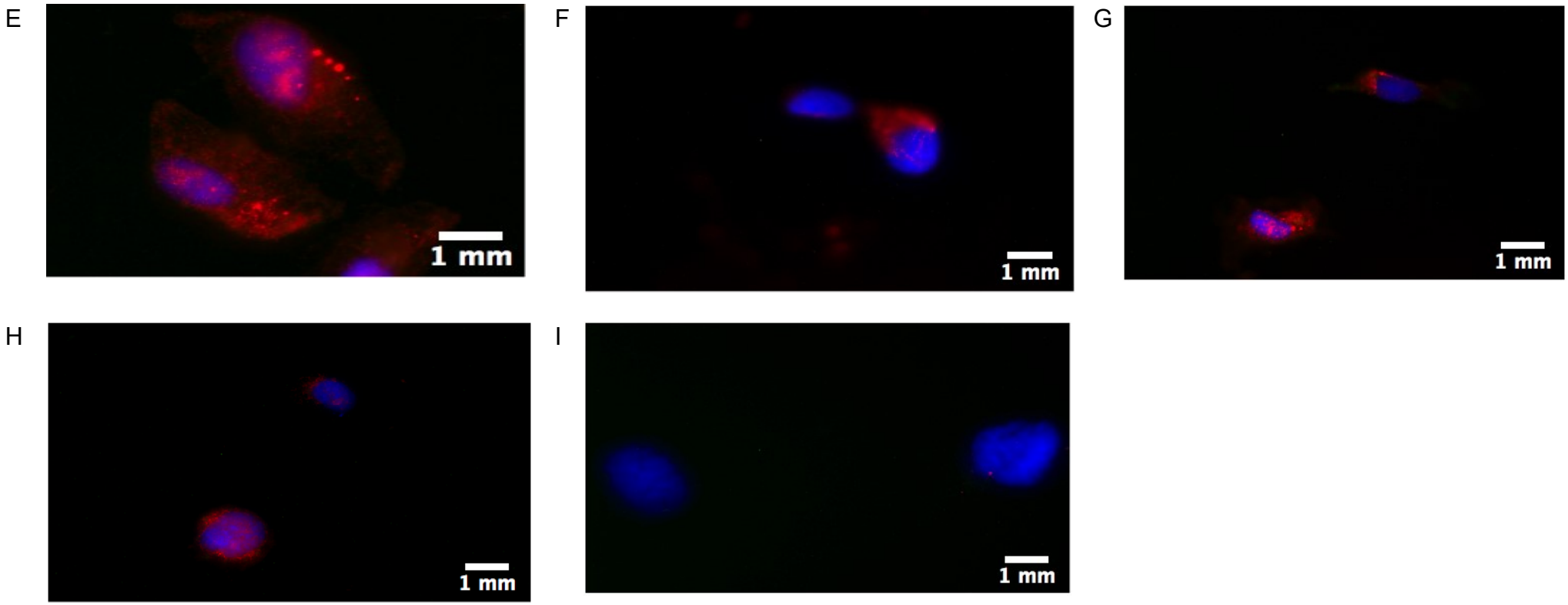


Figure 5.2 Epifluorescence microscopy pictures of P-Selectin (green), E-selectin (red) and DAPI (blue) in HUVEC cells following different treatments. HUVEC cells were incubated with (E)DJ0021. (F)DJ0171 (G) DJ0097 H) DJ0199 and (I) Ticagrelor. For C-I, the cells were treated with P2Y12 inhibitor for 30 minutes prior to 5 minute ADP stimulation and investigated on 40x oil emersion lens..

5.3.2 Effect of thienopyridine treatments on ADP-induced HUVEC cell expression of ICAM-1 and VCAM-1.

TNFα was used again as a positive control in this study due to the well established stimulation of ICAM-1 and VCAM-1 on endothelial cells by this cytokine. The images from the epifluorescence microscopy illustrate the effect of stimulators on HUVEC cell expression of ICAM-1 (green) and VCAM-1 (red), with high expression of both adhesion molecules following TNFα confirming the validity of the method (Fig 5.3A). Expression of VCAM-1 was high in cells following ADP stimulation, yet ICAM-1 expression was significantly lower (Fig. 5.3B).

Interestingly, all novel thienopyridines (and ticagrelor) were found to prevent ADP-induced stimulation of VCAM-1 (Fig. 5.3C and Fig. 5.4 D-I). Obviously there was a notable absence of ICAM-1 expression in the theinopyridine treated cells, but this was no different from the ADP-only treated cells.

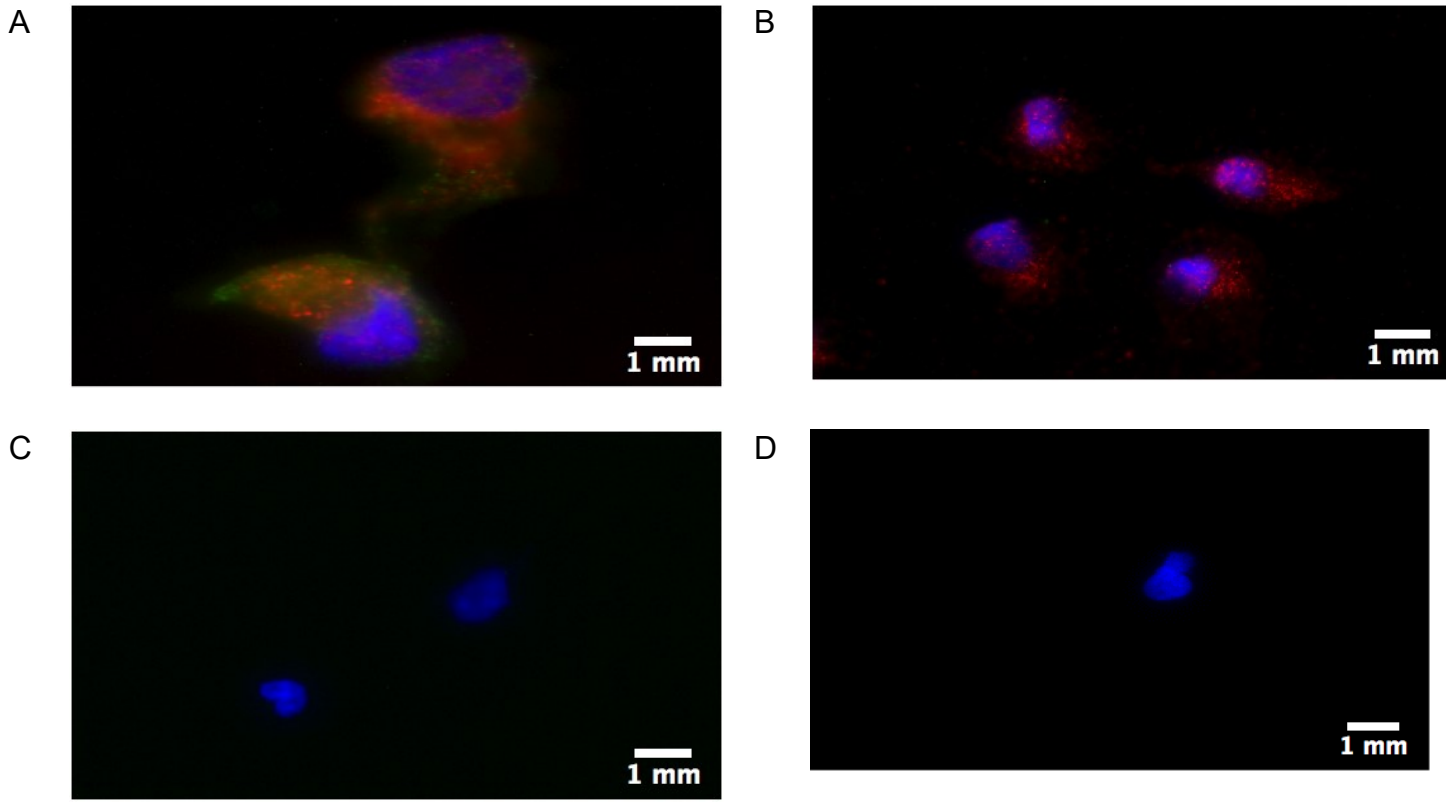


Figure 5.3 Epifluorescence microscopy pictures of ICAM-1 (green), VCAM-1(red) and DAPI (blue) in HUVEC cells following different treatments. HUVEC cells were incubated with (A) TNF-α for 4 hours as positive control. (B) ADP only for 5 minutes, (C) DJ0206. (D) DJ0081. For C-I, the cells were treated with P2Y12 inhibitor for 30 minutes prior to 5 minute ADP stimulation and investigated on 40x oil emersion lens.

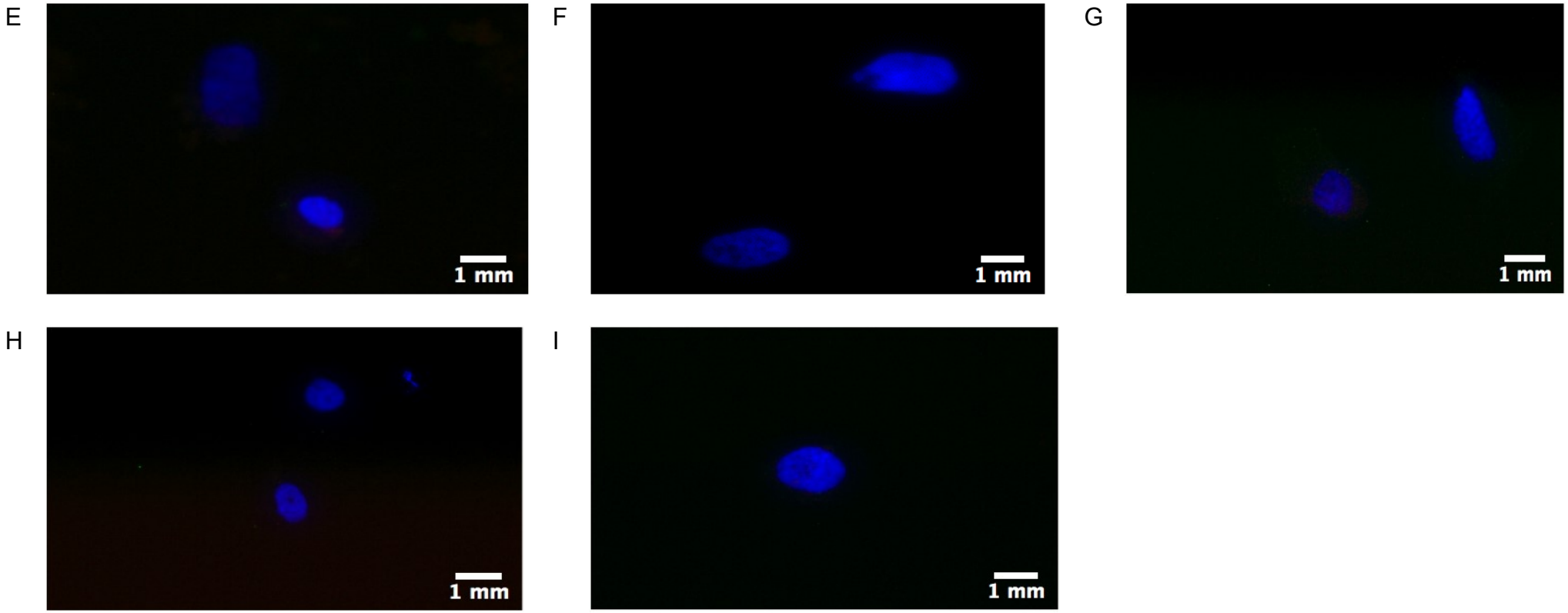


Figure 5.4 Epifluorescence microscopy pictures of ICAM-1 (green), VCAM-1(red) and DAPI (blue) in HUVEC cells following different treatments. HUVEC cells were incubated with (E)DJ0021. (F)DJ0171 (G) DJ0097 H) DJ0199 and (I) Ticagrelor. For C-I, the cells were treated with P2Y12 inhibitor for 30 minutes prior to 5 minute ADP stimulation and investigated on 40x oil emersion lens.

5.4 Discussion

The main finding of this pilot work is that the novel thienopyridine compounds have an effect on the expression of adhesion molecules by HUVEC cells, namely CD62P, CD62E and VCAM-1. It has confirmed that ADP itself stimulates expression of these adhesion molecules, which at present is relatively unclear in the literature. The findings indicate that in the presence of thienopyridines, the recruitment of leukocytes to the site of vascular injury will be impaired (Mark I Furman et al., 1998; Nofer et al., 2010; Kuckleburg et al., 2011) and further indicates that for those patients currently treated with P2Y12 inhibitors such as clopidogrel and ticagrelor, it is not only the platelet responses that will be inhibited, but implies that the anti-haemostatic effects extend to a more global scale. Therefore, these compounds act to suppress the adhesion molecules that contribute to inflammation and the progression of atherosclerosis (Zhang et al., 2002).

It has been demonstrated that ADP effects the production of PGI2 and NO, supressing the release of these platelet inhibitory factors from the endothelial cells (Hess et al. 2009). Evidence also shows that ADP stimulates migration of endothelial cells during the process of angiogenesis and repair of injured tissue (Hess et al., 2009). However, this effect appears to be via interaction with the P2Y1 receptor on endothelial cells, which has down-stream signalling pathways involving the MAPKs ERK1/2, P38 MAPK and JNK (Shen and DiCorleto, 2008). Work on rats has demonstrated that clopidgrel can prevent vascular remodelling and has beneficial effects on the aortic endothelium in hypertensive rats. However, the authors report that the effects do not seem to be directly related to P2Y12 receptors in the vessel (Giachini et al., 2014).

A P2Y12-specific effect of ADP has been shown in vascular smooth muscle cells (VSMC), where vasoconstriction of the vessels following P2Y12 ligation has been observed(Wihlborg et al., 2004). Further work would explore the effects of mainstream P2Y12 inhibitors such as clopidogrel and ticagrelor on VSMCs and also clarify whether similar effects are seen following use of the novel thieopyridines.

Obviously the data are preliminary and extensive further work is required to fully characterise the effects of P2Y12 inhibitors on endothelial cell activation and function. The epifluorescence analysis of adhesion molecule expression presented here provide qualitative data which could be supported by more quantitative methods for protein expression such as flow cytometry, and ELISA.

The research group acknowledged the controversy surrounding the effects of trypsin on expression of adhesion molecule expression. The cells in this work were allowed to adhere to coverslips, fixed and labelled with the antibodies prior to image analysis, rather than employ the method used in many studies that utilises fixing and staining of trypsinised cells in suspension. This method was shown to be a valid method for analysis of E-selectin, VCAM-1 and ICAM-1 in formaldehyde fixed HUVEC monolayer cells (Gräbner et al., 2000).

In conclusion, the study demonstrated that endothelial cells treated with ADP express CD62P, CD62E and VCAM-1. Treatment with ticagrelor and all six novel thienopyridines can inhibit the ADP-induced expression of CD62P, while DJ0171 DJ0199 and ticagrelor showed significant effects on CD62E. ADP did not appear to stimulate ICAM-1. All six

novel thienopyridines as well as ticagrlor completely prevented the ADP-induced expression of VCAM-1. Taken together the results point to the importance of these compounds in the regulation of endothelial dysfunction and the importance of further work in this area.

Chapter 6: Platelet roles outside of haemostasis:

The cancer microenvironment and platelet function.

Chapter 6: Platelet roles outside of haemostasis: The cancer microenvironment and platelet function

6.1 Introduction

The thesis so far has focused on the role of platelets as central players in haemostasis and thrombosis, and looked at the potential for novel thienopyridine compounds to modulate both platelet and endothelial activities.

However, platelets are also central players in innate immunity and inflammation (Morrell et al., 2014; Sabrkhany et al., 2011; Ronnlund et al., 2012; Almog and Klement, 2010), secreting a vast array of cytokines and chemokines that orchestrate the immune response to infection. Factors in the platelet releasate contribute to a wide range of processes such as activation (CD40L, MIP-1α IL-1β), proliferation (TGFβ, PDGF), and chemotaxis (SDF-1, PF4) of leukocyte subsets, while others are involved in adhesion of platelets to leukocytes and endotheial cells (CD62P, GPIbα, vWF) (Reviewed in Morrell et al., 2014). Platelets also contain more than 30 angiogenesis proteins that may contribute to the promotion of new blood vessel growth, particularly in the developing tumour. These include VEGF, bFGF, PDGF, EGF and MMPs, to name a few (Italiano et al., 2008)

This is another area for consideration when applying anti-platelet therapies, as inhibition of platelet signalling will also have an effect on the immunological properties of the platelets. Indeed recent research has shown that the P2Y12 inhibitors clopidogrel and ticagrelor were able to supress systemic inflammation in a human sepsis model

(Thomas et al, 2015). This novel work demonstrated that treatment with these P2Y12 inhibitors was able to reduce platelet-monocyte aggregate formation and peak levels of major pro-inflammatory cytokines, including TNFα, IL-6 and CCL2 that were induced by intravenous injection of *E.coli* endotoxin. The work demonstrates the vital importance of platelets in systemic inflammation and suggests that anti-platelet therapy could be useful in the treatment of systemic inflammatory response syndrome (SIRS) and sepsis.

Moreover, it has been found that platelets act as key mediators of tumour metastasis (Almog and Klement, 2010; Sabrkhany et al., 2011) . Surprisingly, patients with cancer, whether a solid tumour or a haematological malignancy have abnormal haemostatic parameters which may lead to thrombosis or active bleeding (Ronnlund et al., 2012). This indicates that cancer cells themselves or neo-angiogenesis processes may have an effect on platelet function in these patients.

There is a large body of evidence demonstrating that platelets sequester many microenvironmental factors. This could be the reason for abnormal platelet function in the case of cancer patients, where the abnormal cancer cell secretome contributes to a large proportion of the microenvironment (Peterson et al., 2012; Nilsson et al., 2011; G. L. Klement et al., 2009; G. Klement et al., 2004). Some of these factors that can be sequestered in platelets (Table 6.1) have been found to be cancer specific, which has led to great interest in analysing platelet content as an early tumour detection in cancer patients.

Table 6-1 Summary of literature evidencing the uptake of tumour-derived proteins by platelets in different cancers

Author	Cancer type	Sequestered tumour-derived factors by platelets
Klement et al., 2009	Malignant animal – breast cancer	VEGF
Nilsson et al., 2011	Glioma and prostate cancer	Tumour associated RNA (EGFRVII and PCA3)
Kerr et al., 2010	Human prostate cancer cell	TGF-β_1, VEGF, MCP-1,MMP-2, RANK, RANKL and TIMP-1.
Fu et al., 2015	Non-small cell lung cancer (NSCLC)	VEGF
Benoy et al., 2002	Breast cancer	VEGF
Klement et al., 2004	Human tumour injected in mice	VEGF, bFGF, PDGF, BDNF, endostatin
Peterson et al., 2012	Colorectal cancer	VEGF, PDGF and PF4
Cervi et al., 2008	Liposarcoma	PF4

A number of studies report significant changes in cytokine profiles and growth factor levels in the serum of cancer patients compared with healthy people. Interestingly, IL-6, TNF-α and VEGF have been highlighted as those factors most commonly over-expressed in cancer patients. It has been suggested that tumour derived IL-6 has a role in controlling VEGF level in the platelets of breast cancer patients which demonstrates the link between the serum level of IL-6 and VEGF concentration in platelets (Benoy et al., 2002).

Other studies have reported the percentage of thrombosis and bleeding in cancer patients (Table 6.2). Surprisingly, it has been reported that VTE incidence in cancer patients is seven times higher than that in a healthy population (Falanga et al., 2013). In contrast, active bleeding has also been seen in cancer patients, and is considered as a major cause of death in 10% of solid tumour patients.

Table 6-2 Collective evidence of haemostatic abnormalities in cancer patients.

Author	Cancer type	No. of patients	Haemostatic abnormality	Proposed cause of haemostatic abnormality.
Klasters et al., 1972	Different cancer (leukemia, breast and lung cancer)	157	Severe haemorrhage - 11.4% died as a direct result	Severe thrombocytopenia
	Acute leukemia	27	Severe haemorrhage - 30% died as a direct result	
Falanga et al., 2013	Myeloproliferative neoplasms	NA	10%-40% Thrombosis ATE	Unknown
	HL, NHL and MM	NA	The highest incidence of VTE (thrombosis) among haematological malignancy	Unknown
Belt et al., 1978	Solid tumors	718	10.4% presented with active bleeding	Multifaceted: 49.3% due to drug induced thrombocytopenia, 33.3% due to tumor-invasion, 9.3% due to DIC and 8% was not related to malignant neoplasm neither chemotherapy.

Table 6-2 Collective evidence of haemostatic abnormalities in cancer patients.

Cartoni et al., 2009	Haematological malignancies (leukemia, lymphoma, MM, MDS, MPNs).	469	23% presented with major bleeding	Main reason for bleeding was due to low platelet count (<20×10^9/L).
	Acute myeloid leukemia	69	54% presented with active bleeding	
	Blast crisis (in CML?)	56	46% presented with active bleeding	
	ALL	39	46% presented with active bleeding	
	MM	39	13% presented with active bleeding	Interference of serum proteins with platelet activity and coagulation factors
Caruso et al., 2010	Lymphoma	18081 (meta-data)	Incidence of thrombosis as 6.4%	Unknown
	NHL	1180	7% thrombosis	Unknown
	HL	177	5.6% thrombosis	Unknown
Goldschmidt et al., 2003	CNS lymphoma	42	59.5% presented with VTE and 7% died due to VTE	Unknown

We decided to use a Hodgkin Lymphoma cell model to study the interplay between the cancer cell secretome and platelet function. Many cytokines are released from lymphoma cells at abnormal levels, such as IL-6, TNF α, IL-1β, IL-5, IL-9, M-CSF and TGF-β_1 (Hsu et al., 1993), where they seem to have a role in disease progression. It has been demonstrated that the level of these cytokines is higher in circulation in lymphoma patients compared to healthy individual (da Silva et al. 2015).

Hodgkin Lymphoma is one of the most common lymphomas in the UK, with 2000 new cases per year (CRUK, 2016). Although, the management of this disease has improved with the introduction of newer treatment approaches, both thrombosis and bleeding are difficult to manage in these patients. The incidence rate of thrombosis in lymphoma patents varies between 3-13%, and can reach up to 60% in lymphoma cases with primary brain metastasis (Caruso et al., 2010).

The aim of this part of the study was to further clarify the effect of the lymphoma microenvironment on platelet function via the use of Lymphoma cell lines, recombinant cytokines and platelets obtained from healthy individuals.

The aim was achieved via the following objectives:

- To investigate the effect of the Hodgkin Lymphoma cell line, L1236, secretome on healthy platelet activation and function.
- To determine the effects of recombinant cytokines on healthy platelet activation and function.

6.2 Methods

6.2.1 Specimen collection

Blood Samples were collected from healthy volunteers in citrated vacutainers and PRP/PPP was isolated as described in section 2.3.

6.2.2 Cell culture

The Hodgkin lymphoma cell line L1236 was used in this study. Cells were routinely passaged and maintained in RPMI media, supplemented with 15% FBS at 37^0C in a humidified environment of 5% CO_2 in air. Cell viability was assessed using the Trypan blue exclusion method on a haemocytometer, and experiments were only performed when cell viability was >95%.

Cells, in a total volume of 3ml of the media were seeded into 6-well plates at varying densities as shown in table 6.3 and incubated at different incubation times. Each concentration was run in duplicate.

Table 6-3 Cell culture concentration at different incubation times

Cell Conc.	Incubation time			
1×10^5 cell/ml	24 Hours	48 Hours	72 Hours	96 Hours
5×10^5 cell/ml	24 Hours	48 Hours	72 Hours	96 Hours
1×10^6 cell/ml	24 Hours	48 Hours	72 Hours	96 Hours

Following incubation, each sample was removed from the well, centrifuged at 500 g for 5 minutes at 22°C and the supernatant (conditioned media (CM)), was removed and stored for later use at -80°C.

6.2.3 Treating platelet with conditioned media

CMs from three different cell concentrations that were incubated for 24, 48, 72, 96 hours, were taken from -80˚C freezer and thawed at room temperature for 30min. A 225µl volume of CM was added to an equal volume of PRP and incubated for three hours at room temperature (RT) to determine the effect of CM from lymphoma cells on healthy platelets. Control samples contained 225µL PRP and 225µL fresh RPMI media.

6.2.4 Treating platelets with IL-6 and TNF-α

As many cancer cells (including lymphoma cells) secrete high levels of IL-6 and TNF-α (Hsu et al., 1993), it was proposed that these cytokines, present in the CM, could affect platelet aggregation. Therefore, PRP from healthy subjects was incubated with different concentrations of IL-6 or TNF-α (Invitrogen, UK). The same concentrations for both cytokines was used (1000,100,10,1,0.1 and 0ng/ml). A 400µl volume of PRP was incubated with 50µl of the relevant cytokine at RT for 3 hours. LTA was performed using ADP or Collagen as described in section 2.7. The control in this experiment was incubating 400µl of PRP with 50µL tyrodes buffer prior to LTA.

6.2.5 Light transmission aggregometry (LTA)

LTA was performed as described in section 2.7. Briefly, 450µl of PRP-CM samples or cytokine-treated PRP samples where stimulated with 50µl of ADP or Collagen used at 10µM and 2µg/ml respectively. PPP was used as the LTA 100% control.

6.2.6 Flow cytometry

Expression of the activation markers CD62P and activated fibrinogen receptor using anti-CD62P and PAC1 were was tested. PRP was incubated with CM or IL-6/TNFα as before but the volumes used for the testing in this methodology was different. An 80µL volume of PRP was incubated with 10µL cytokine at different concentrations (as above). Following incubation, 4µL fluorescence isothiocyanate (FITC)- PAC-1 and 4µL phycoerythrin (PE)-CD62P were added with 10µL ADP as the agonist. 100µL paraformaldehyde (PFA) 4% was also added to fix the cells followed by 300µL Dulbecco's phosphate buffered saline (DPBS). Cells were the analysed immediately on a FACSVerse Flow Cytometer using BD FACSSuite software (Becton Dickinson, UK)

6.2.7 Statistical Analysis

GraphPad Prism version 5.0 was used for analyses. Statistical analysis was performed using one-way ANOVA with Dunnett's post-hoc test. A significant result was represented as $p < 0.05$.

6.3 Results

6.3.1 Effect of conditioned media on platelet aggregation

6.3.1.1 Platelet aggregation Using ADP as the agonist

Platelet rich plasma (PRP) was incubated with CM collected from L1236 cells incubated at varying cell densities for varying times. There was a difference in maximum platelet aggregation (MaxA) between PRP incubated with CM and the control samples when they were triggered by ADP. The representative aggregometry trace shows a decrease in aggregation from control (blue line) when PRP was treated with CM from cells cultured for varying times (24, 48 and 72 hours) (Fig. 6.1). Figure 6.2 shows that the decrease in aggregation was independent of culture time before CM collection or concentration of cells used for culture.

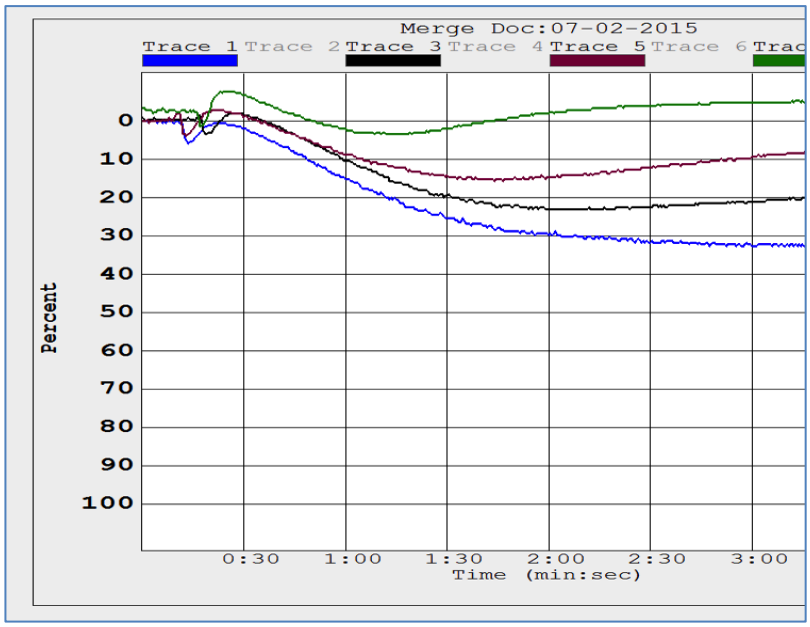


Figure 6.1 Representative Aggregometry Trace following incubation of PRP with CM from cultured L1236 cells - Blue: Control, Black: CM from 48h culture, Purple: CM from 72h Culture, Green: CM from 96h Culture.

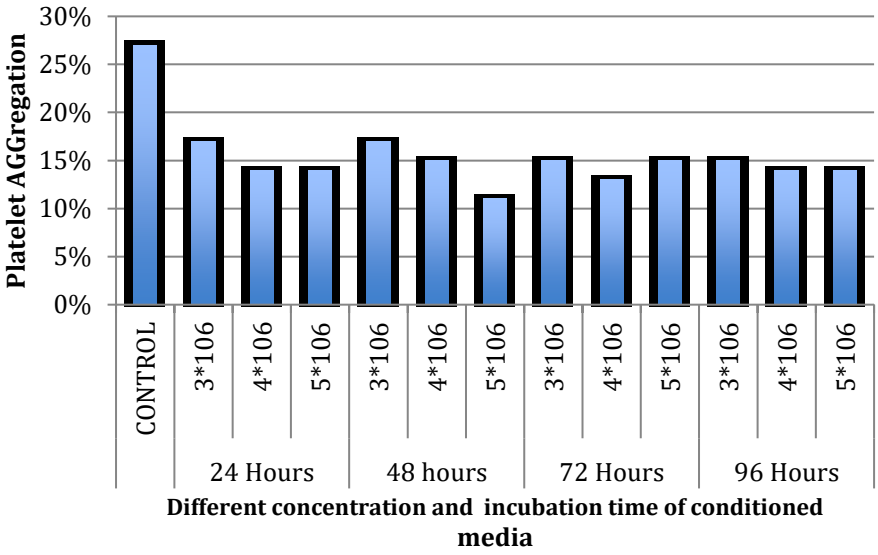


Figure 6.2 Platelet Aggregation in PRP from healthy individuals treated with CM from cells seeded at varying densities for varying times. Data is expressed at mean of two independent samples n=1.

6.3.1.2 Platelet aggregation using collagen as the agonist

PRP incubated with CM was analysed by LTA as before but collagen was used as the agonist. This time only the effect of L1236 culture time was studied. Similarly to the results observed using ADP as the agonist, there was a significant decrease (*P*=0.002) in MaxA in platelets incubated with CM compared to the control sample following stimulation with collagen (Fig 6.3). The decrease in platelet aggregation was seen in all samples incubated with CM (Fig 6.4).

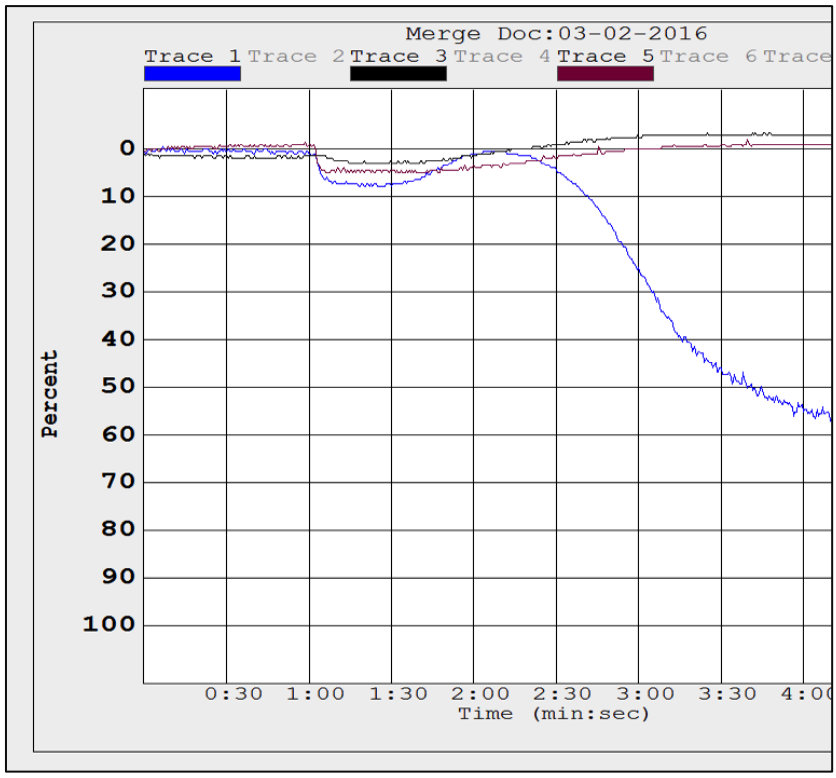


Figure 6.3 Representative Aggregometry Traces from a single participant. PRP was pre-incubated for three hours with conditioned media (or tyrode's buffer) collected from $1x10^6$ L1236 cells before stimulation with Collagen (1 µg/ml) for 5 minutes. Blue Line: Control, Black Line: 48h culture, Purple Line: 72h Culture.

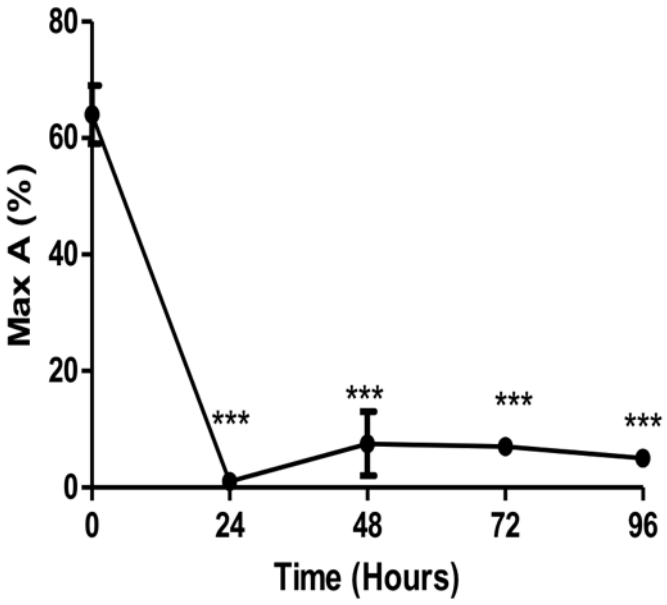


Figure 6.4 Maximum Platelet Aggregation in PRP from healthy individuals treated with CM from cells seeded at 1x106 cells/well for varying culture times. Data is presented as mean ± SEM of n=3. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test. Statistical differences are highlighted on the graph. *** Represents p<0.0001.

6.3.2 Effect of conditioned media on platelet activation

6.3.2.1 Analysis of platelet activation using ADP as the agonist

The activation status of healthy platelets following treatment with CM was assessed by analysis of PAC-1 binding to the activated fibrinogen receptor, and CD62P (P-selectin) expression. Representative flow cytometry histograms show the marked inhibition of platelet activation There was significant inhibition in ADP-induced platelet activation when platelet had been pre-treated with CM (Fig 6.5).

The activation status was assessed following treatment with CM that had been collected from L1236 cells cultured at a range of cell concentrations ($3x10^6$, $4x10^6$ and $5x10^6$ cells/ml) at a range of time points (24, 48, 72 and 96 hours). The collected CM was used to treat PRP from three independent donors, with each sample treated twice. The collective data demonstrated that the lymphoma secretome significantly inhibited platelet activation at all cell concentrations and all culture durations, evidenced by both a lower CD62P expression (Fig 6.6) and PAC-1 binding (Fig 6.7).

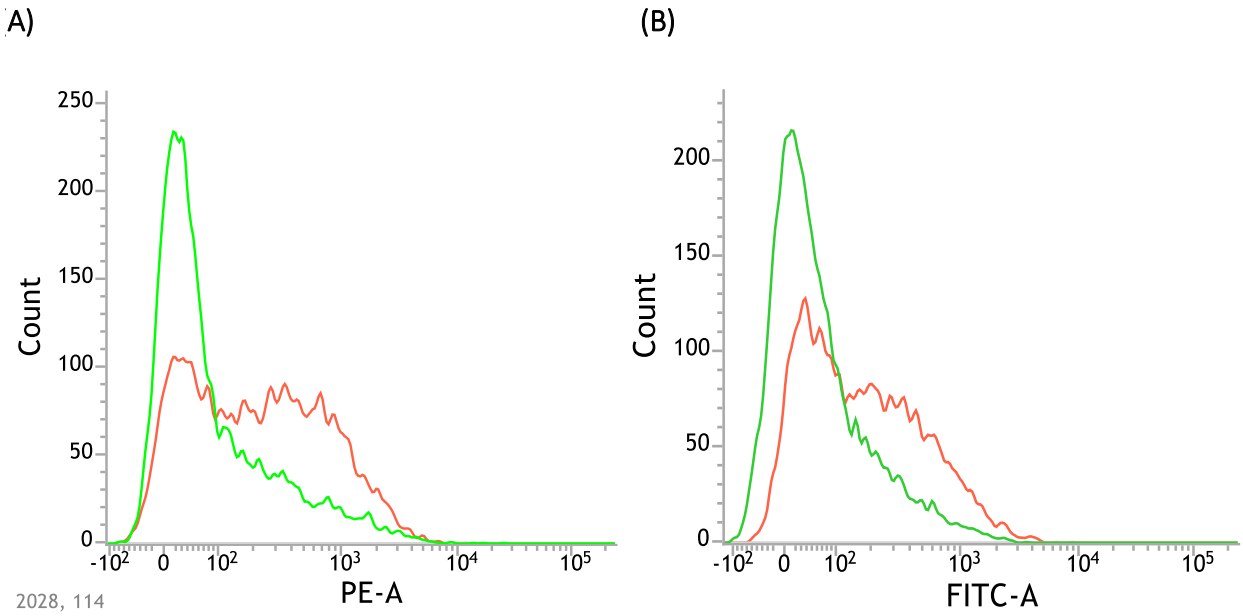


Figure 6.5 Representative FACS histograms for CD62P expression and PAC-1 expression, respectively, on untreated platelets (red) and platelets following 1-hour culture with conditioned media from $1x10^6$ cells/ml L-1236 cells ($p<0.01$) (green). Healthy platelets were stained n=3 by (A) PE-A anti-CD62P (B) FITC-A anti-PAC-1 for 20 minutes following stimulation with ADP (10 µM). The data represents 10 000 events.

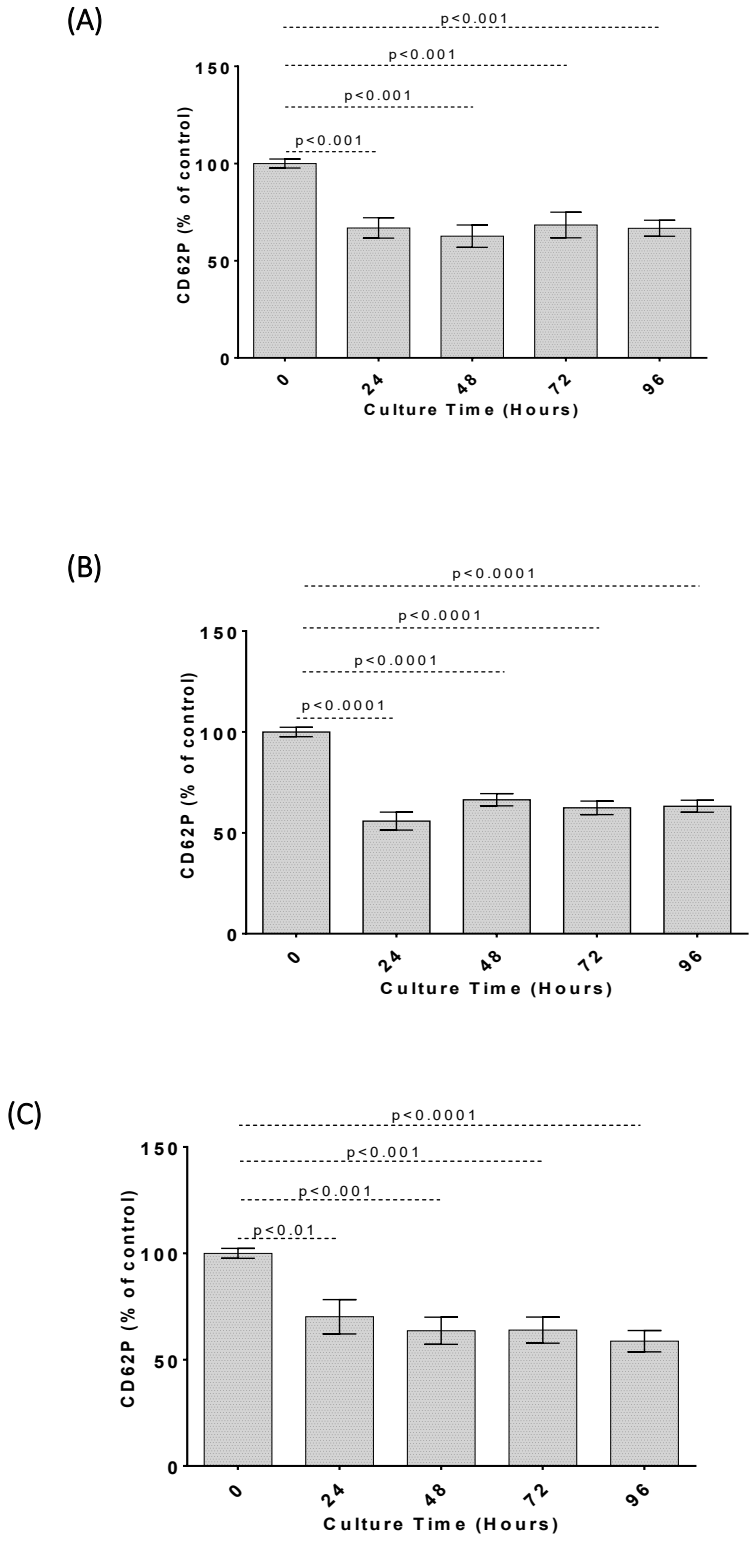
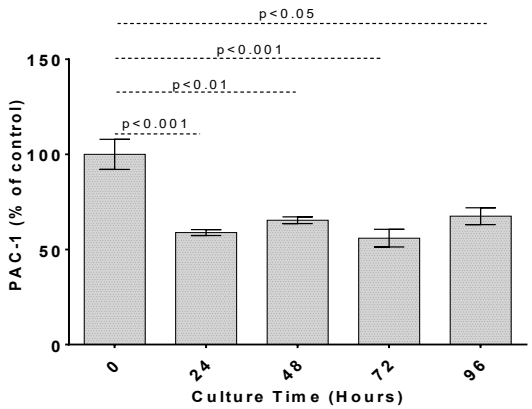
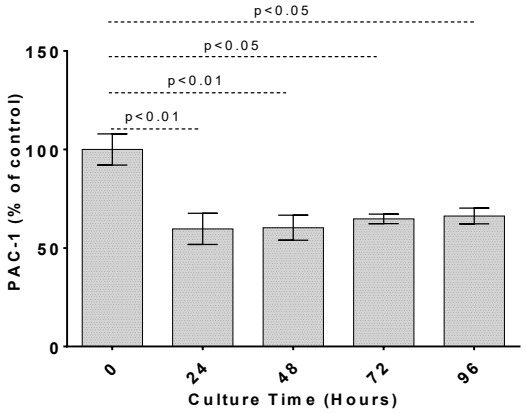


Figure 6.6 ADP-induced CD62P expression (% of control) in healthy platelets after 1-hour culture with conditioned media from **(A)** $3x10^6$ cells/ml L-1236 cells ($p<0.0001$), **(B)** $4x10^6$ cells/ml L-1236 cells ($p<0.0001$) **(C)** $5x10^6$ cells/ml L-1236 cells ($p=0.0001$). Data is represented as mean $\pm$ SEM. Data was produced from three donors, n=3. Statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test and significant differences are indicated.

(A)



(B)



(C)

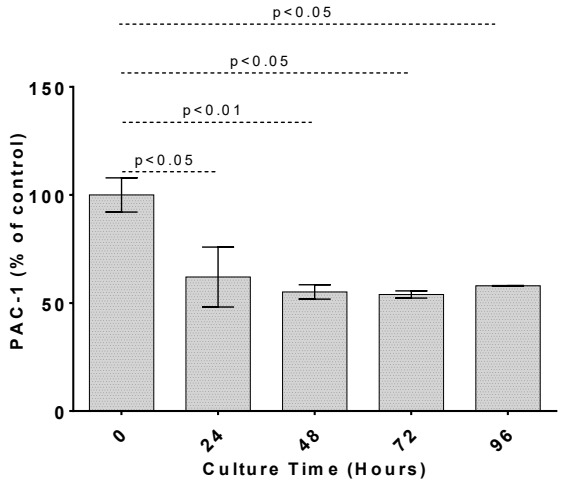


Figure 6.7 ADP-induced PAC-1 binding (% of control) in healthy platelets after 1-hour culture with conditioned media from (A) 3x106 cells/ml L-1236 cells (p=0.0008) (B) 4x106 cells/ml L-1236 cells (p=0.0076) (C) 5x106 cells/ml L-1236 cells (p=0.0069). Data is represented as mean + SD. Data was produced from three donors (n=3). Statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test and significant differences are indicated.

6.3.2.2 Analysis of platelet activation using collagen as the agonist

The activation status was assessed following treatment with CM that had been collected from L1236 cells cultured at $5x10^6$ cells/ml at a range of time points (24, 48, 72 and 96 hours). The collected CM was used to treat PRP from three independent donors, with each sample treated twice.

Collagen-induced expression of CD62P significantly decreased when PRP was incubated with the lymphoma secretome that had been collected after 96 hours of culture (Fig 6.8). Also, PAC-1 binding decreased significantly in samples incubated with the lymphoma secretome that had been collected after 72 or 96 hours (Fig 6.9). This illustrates the inhibitory effect of the secretome on platelet activation, and suggests a mechanism for bleeding tendencies observed in some Lymphoma patients.

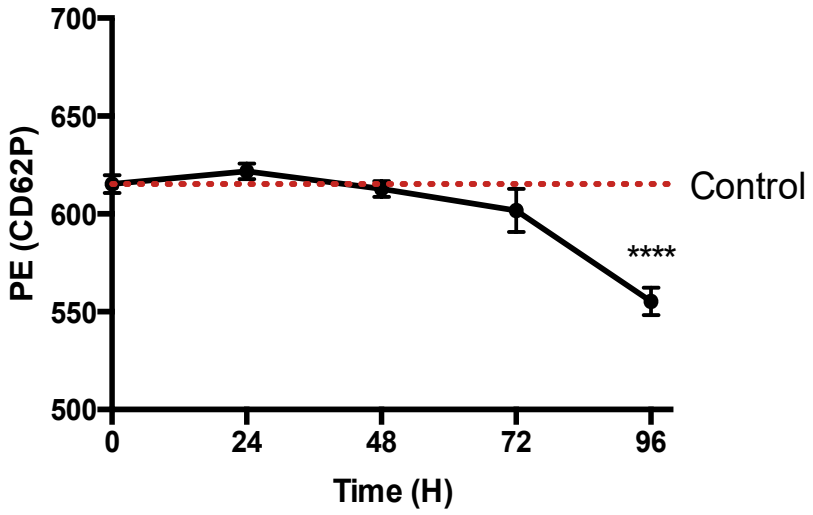


Figure 6.8 Collagen-induced CD62P expression (% of control) in healthy platelets after 1-hour culture with conditioned media from 5x106 cells/ml, L-1236 cells (p=0.0001) collected after varying culture times. Data is represented as mean + SEM. Data was produced from three donors (n=3). Statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test and significant differences are indicated. **** represents P<0.0001.

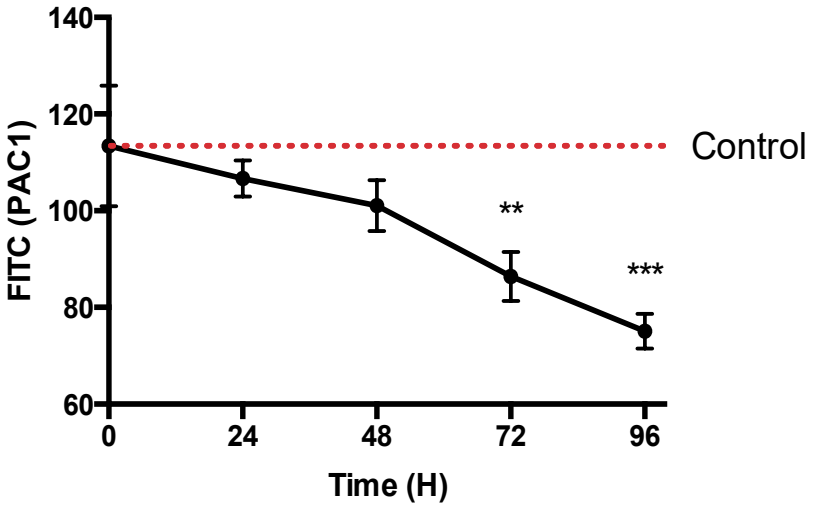


Figure 6.9 Collagen-induced PAC-1 binding (% of control) in healthy platelets after 1-hour culture with conditioned media from 5x106 cells/ml, L-1236 cells (p=0.0001) collected after varying culture times. Data is represented as mean + SEM. Data was produced from three donors (n=3). Statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test and significant differences are indicated. **** represents P<0.0001.

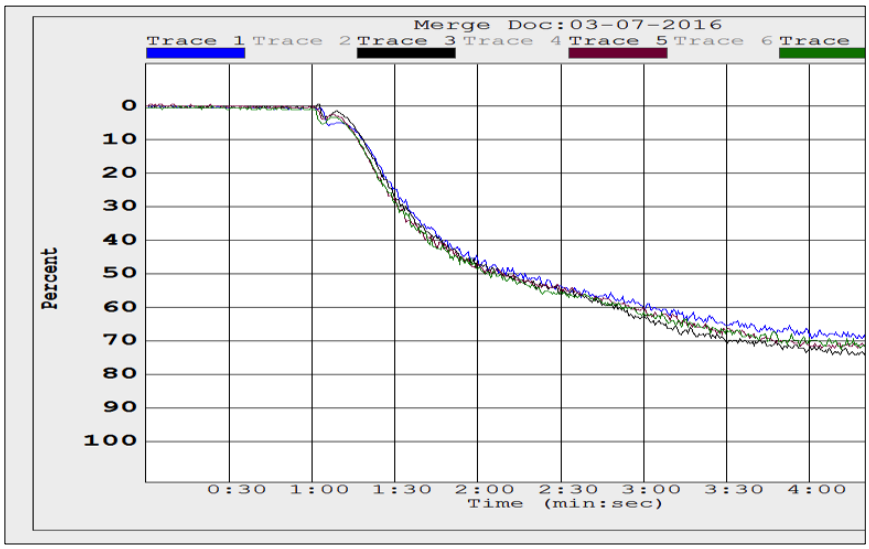
6.3.3 The Effect of IL-6 and TNF-α on Platelet Aggregation.

IL-6 and TNFα are elevated in the serum of lymphoma patients and are secreted by lymphoma cells, including L1236 *in-vitro*. As there is significant inhibition in ADP- and collagen-stimulated MaxA of platelets incubated with CM from L1236, it was proposed that IL-6 and TNFα may be exerting this effect. So two different experiments were conducted treating PRP with IL-6 or TNFα and using ADP as the platelet agonist.

6.3.3.1 Platelet Aggregometry Studies using ADP as the Agonist

There was no significant difference in ADP-stimulated MaxA after treating platelets with different concentrations of IL-6 (Fig 6.10) or TNF-α (Fig 6.11), when compared with control (Tyrode's buffer-treated) samples. The LTA traces show there is no difference in platelet aggregation, with the maximum platelet aggregation at approximately the same level in CM-treated samples as the control sample aggregation (≈70% and 80% respectively).

(A)



(B)

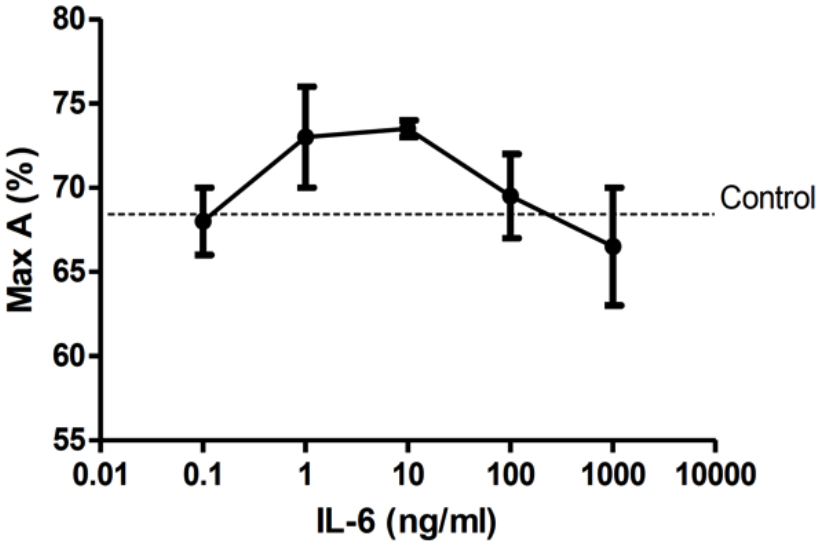
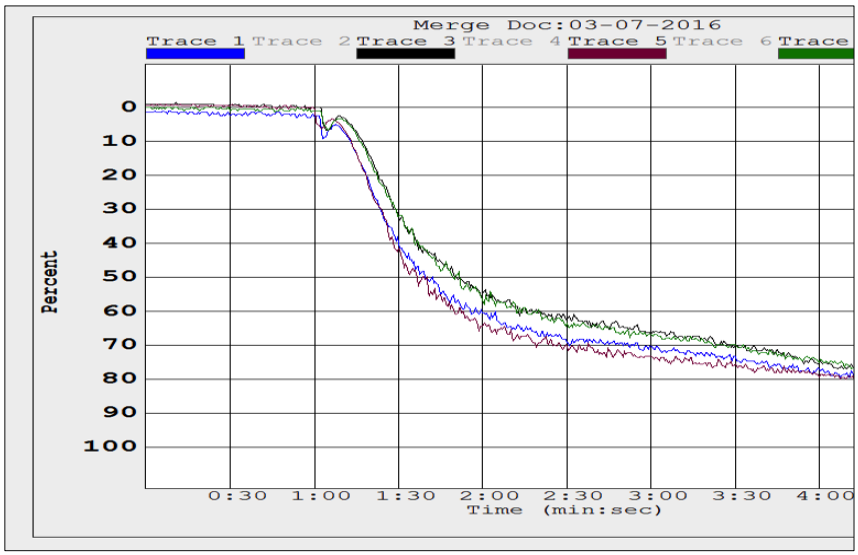


Figure 6.10 (A) Representative Platelet aggregation trace from a single healthy participant, following treatment of PRP with different concentrations of IL-6 in a dose dependent manner, followed by stimulation with ADP (10 µM). Blue Line: Control, Black Line: 0.1ng/ml, Purple Line: 1.0 ng/ml, Green Line: 10ng/ml). (B) Collective data from three healthy participants (n=3). Data is represented as mean ± SEM. One-way ANOVA with Dunnett's post-hoc test revealed no significant differences in MaxA (%) from control (tyrode's buffer-treated platelets).

(A)



(B)

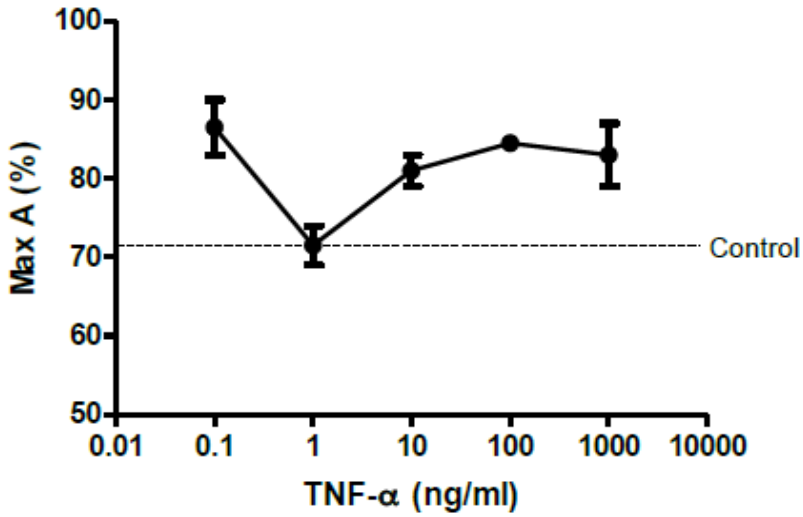
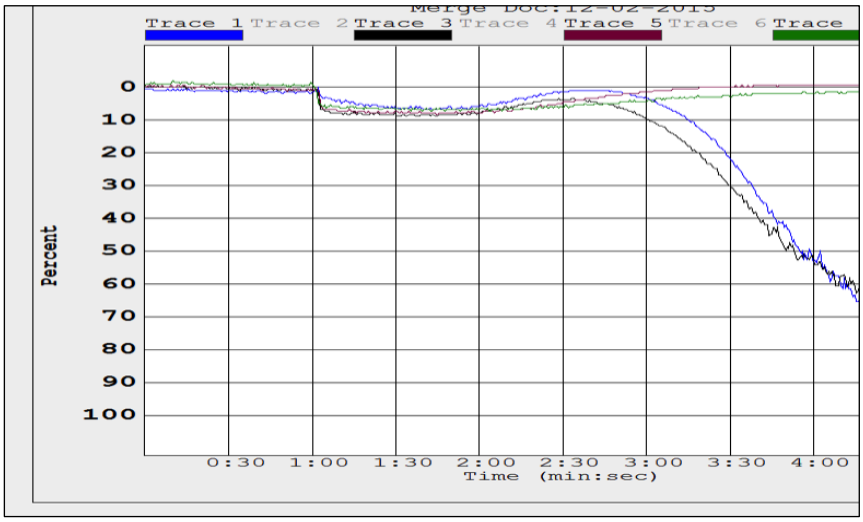


Figure 6.11 (A) Representative Platelet aggregation trace from a single healthy participant, following treatment of PRP with different concentrations of TNF-α in a dose dependent manner, followed by stimulation with ADP (10 µM). Blue Line: Control, Black Line: 0.1ng/ml, Purple Line: 1.0 ng/ml, Green Line: 10ng/ml). (B) Collective data from three healthy participants (n=3). Data is represented as mean ± SEM. One-way ANOVA with Dunnett's post-hoc test revealed no significant differences in MaxA (%) from control (tyrode's buffer-treated platelets).

6.3.4 Platelet Aggregometry Studies using Collagen as the Agonist

In contrast to the results observed using ADP as the agonist, when stimulating IL-6- or TNF-α -treated PRP with collagen, there was a significant difference in aggregation when compared with control (Tyrode's buffer-treated) samples. The significant difference was dose-dependent for both IL-6 (Fig 6.12) and TNF-α (Fig 6.13).

(A)



(B)

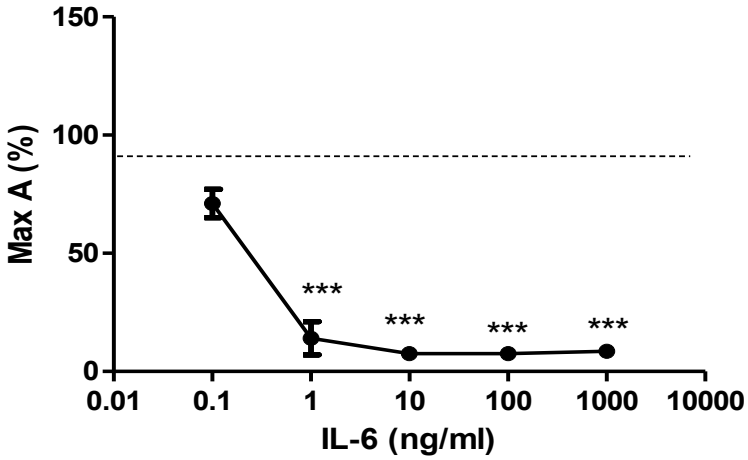
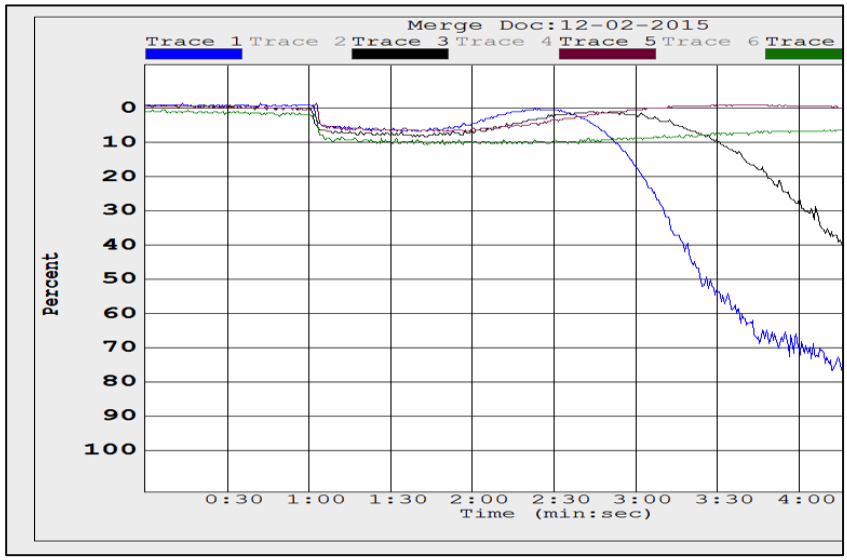


Figure 6.12 (A) Representative Platelet aggregation trace from a single healthy participant, following treatment of PRP with different concentrations of IL-6 in a dose dependent manner, followed by stimulation with Collagen (1 µg/ml). Blue Line: Control, Black Line: 0.1ng/ml, Purple Line: 1.0 ng/ml, Green Line: 10ng/ml). (B) Collective data from three healthy participants (n=3). Data is represented as mean ± SEM. One-way ANOVA with Dunnett's post-hoc test revealed no significant differences in MaxA (%) from control (tyrode's buffer-treated platelets).

(A)



(B)

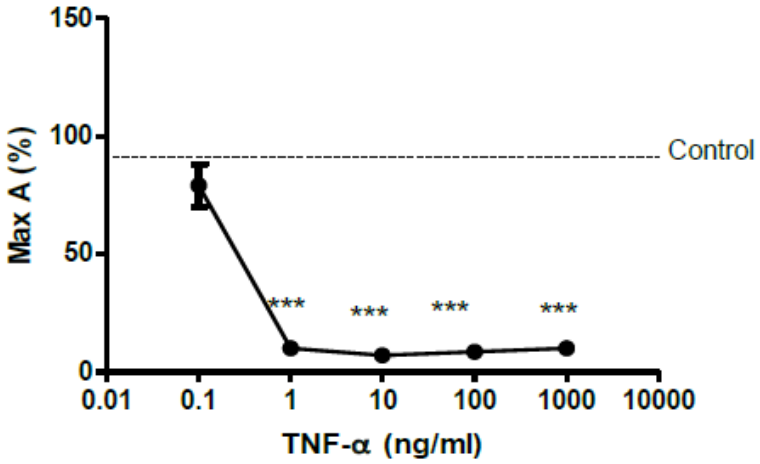


Figure 6.13 (A) Representative Platelet aggregation trace from a single healthy participant, following treatment of PRP with different concentrations of TNF-α in a dose dependent manner, followed by stimulation with Collagen (1 µg/ml). Blue Line: Control, Black Line: 0.1ng/ml, Purple Line: 1.0 ng/ml, Green Line: 10ng/ml). (B) Collective data from three healthy participants. Data is represented as mean ± SEM. One-way ANOVA with Dunnett's post-hoc test revealed no significant differences in MaxA (%) from control (tyrode's buffer-treated platelets).

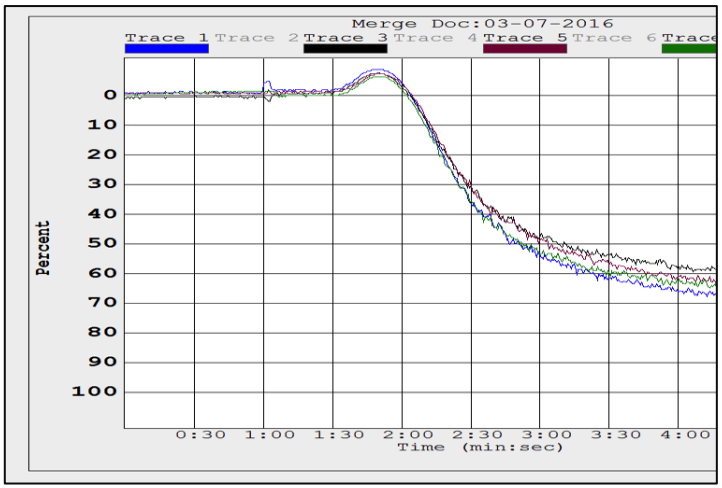
6.3.5 Verification of IL-6/TNFα effects on collagen-induced platelet aggregation

As platelet aggregation was not affected by IL-6 or TNF-α when ADP was used as the agonist, further confirmation was required that collagen-induced aggregation was affected by these cytokines. However, the results appeared inconsistent every time with every donor. Therefore, it was proposed that there may be a stability issue with the collagen itself that needed resolving. Rather than diluting the collagen to the required concentration (1mg/ml) in Tyrode's solution, it was decided to add neat collagen directly to the sample at a 1:1000 ratio in PRP (final concentration of 1mg/ml). This prevented the collagen from forming aggregates in the Tyrode's buffer and degrading.

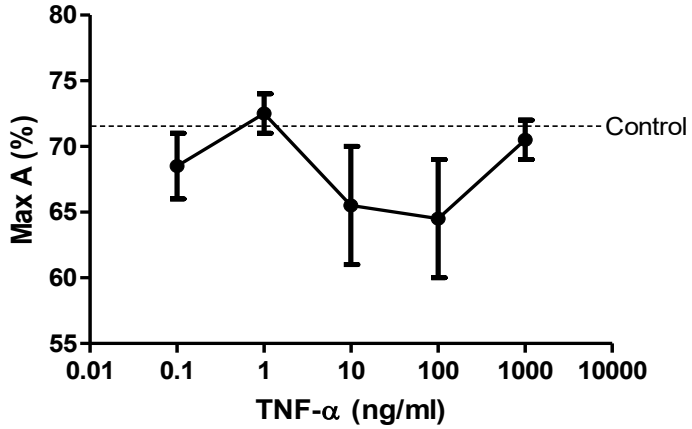
6.3.6 Repeating platelet aggregation following IL-6 and TNFα, using non-pre-diluted collagen.

There was no significant difference in platelet MaxA aggregation after repeating the experiments involving incubation of platelets with IL-6 and TNFα, and stimulating with non-pre-diluted collagen (Fig 6.14). Although platelet aggregation appeared to decrease when the platelets were incubated with 10 and 100ng/ml TNF- α, MaxA returned close to levels in the control samples when treated with 1000ng/ml. Furthermore, the platelet aggregation traces did not identify any difference between the control and treated samples with TNF- α and IL-6.

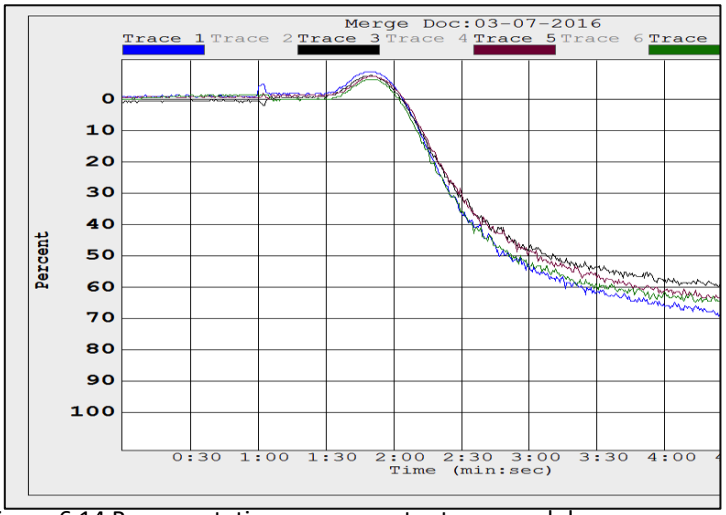
A



B



C



D

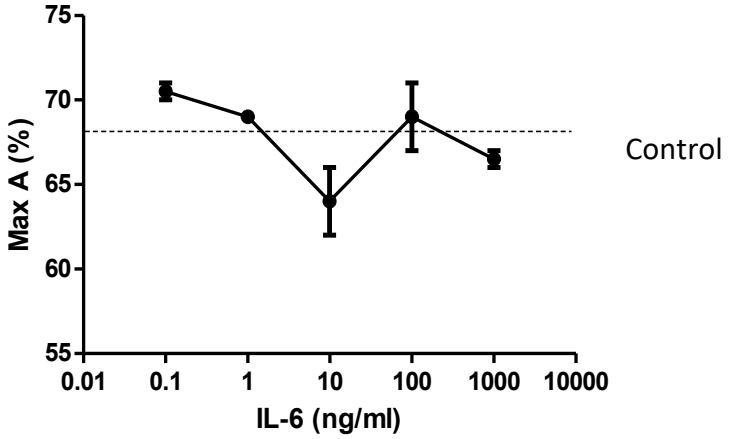


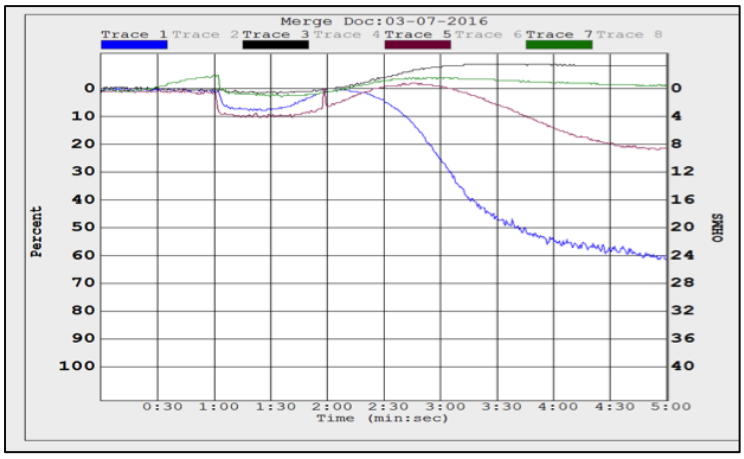
Figure 6.14 Representative aggregometry traces and dose response curves in response to TNF- α (A and B) and IL-6 (C and D), after stimulation with non-pre-diluted collagen. For aggreometry traces, Blue: Control, Black: 0.1ng/ml, Purple: 1.0 ng/ml, Green 10ng/ml) (B &D) There was no significant difference in Max platelet aggregation of incubated platelets with different concentration of IL- and TNF-α. statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test.

6.3.7 Repeating platelet aggregation following L1236 CM, using non-pre-diluted collagen.

Re-testing collagen-induced platelet aggregation following incubation with CM was important to confirm that earlier results were significant and not due to collage instability.

The results still showed significant differences between PRP incubated with with CM of L1236 and control samples, verifying the results seen earlier (Fig. 6.15). This shows that the tumour (specifically lynphoma) secreome has a significant effect on platelet aggregation which may be responsbile for the increased bleeding tendency often observed in the cancer setting.

A



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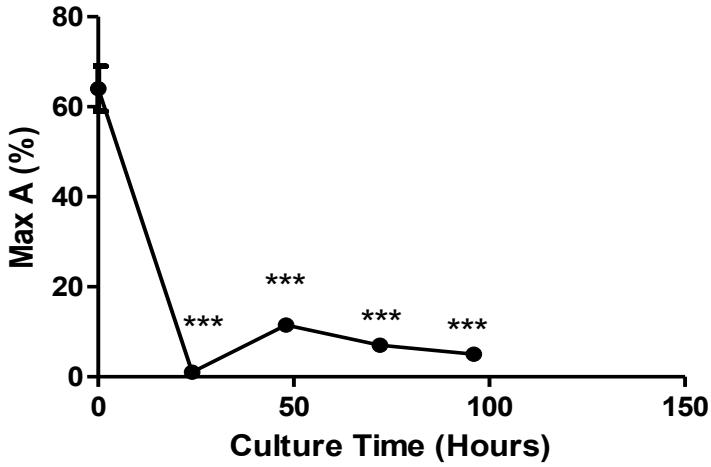

Figure 6.15 Platelet aggregation following incubation with CM from L1236 cells, and stimulated with non-pre-diluted collagen. (A) Represenative aggregmetry trace (Blie: Control, Black: 48h Culture CM, Purple: 72h Culture CM, Green: 96h Culture CM). (B) Collective data fro three healthy participants. Data is represented as mean ± SEM. One-way ANOVA with Dunnett's post-hoc test revealed no signifciant differnce in MaxA (%) from control (Tyrode's buffer-trested platelets).

6.3.7.1 Repeating platelet activation following L1236 CM using non-pre-diluted collagen

Collagen-induced CD62P expression was significantly lower following incubation with CM collected after 96 hour culture of L1236 cells (Fig 6.16) when compared to expression in control samples. Also, Collagen-induced PAC1 binding was also significantly lower following incubation with CM collected after 72 and 96 hour culture of L1236 cells (Fig 6.17). These data illustrate the inhibitory effect of the tumour (specifically lymphoma) secretome on platelet activation, which again, may be responsbile for the increased bleeding tendency often observed in the cancer setting.

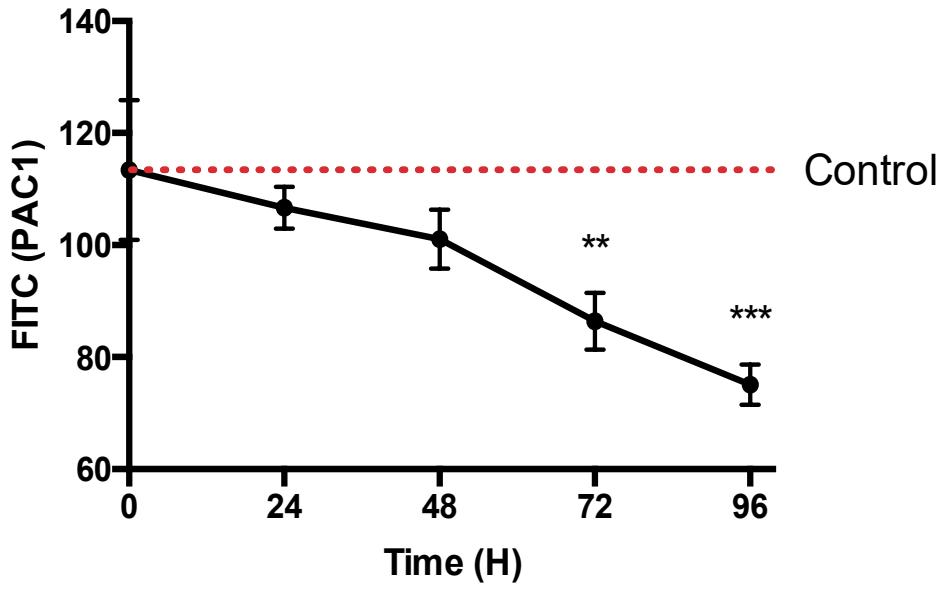

Figure 6.16 Collagen-induced PAC-1 expression (% of control) in healthy platelets after 1-hour culture with conditioned media from $5x10^6$ cells/ml, L-1236 cells (p=0.0001) collected after varying culture times. Data is represented as mean ± SEM. Data was produced from three donors (n=3). Statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test and significant differences are indicated. **** represents P<0.0001

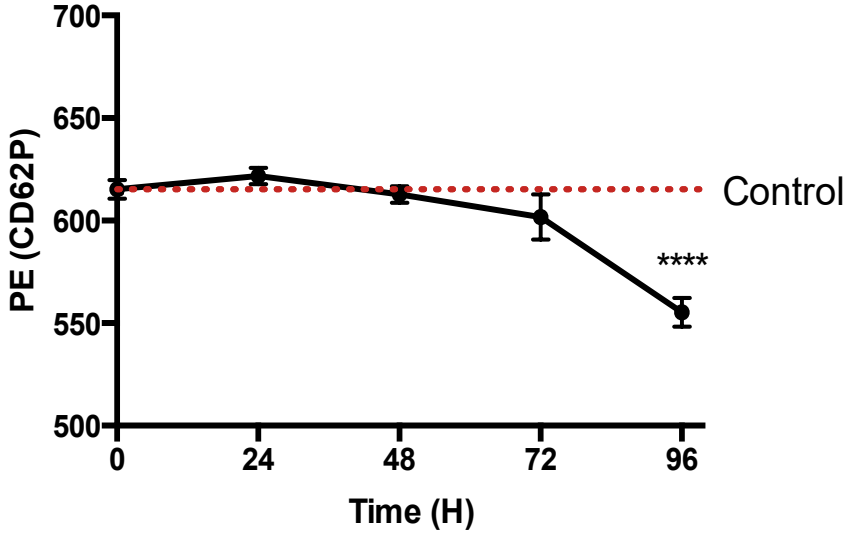

Figure 6.17 Collagen-induced CD62P expression (% of control) in healthy platelets after 1-hour culture with conditioned media from 5x106 cells/ml, L-1236 cells (p=0.0001) collected after varying culture times. Data is represented as mean + SEM. Data was produced from three donors (n=3). Statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test and significant differences are indicated. ** represents P<0.01, *** represents p<0.001.

6.3.8 Discussion

Platelets have been shown to sequester tumour-derived proteins that are secreted from cancer cells such as lymphoma cells into the microenvironment (Klement et al., 2009; Nilsson et al., 2011; Kerr et al., 2010). The effect of these proteins on the haemostatic function of platelets has, so far not been investigated.

The initial data from this project showed that incubating healthy platelets with a tumour secretome (conditioned media from Hodgkin Lymphoma cells) significantly inhibits platelet aggregation and activation potential, upon stimulation with either ADP or collagen (Fig 6.1-6.9). Platelets were inhibited after just three hours of CM-treatment, and the effect was observed irrespective of time of lymphoma cell culture or L1236 seeding density. This would account for the increased risk of bleeding seen in a proportion of lymphoma patients.

There are a large number of factors known to be released by lymphoma cells in culture and many of these, including IL-6, TNFα, IL-5, IL-9, IL-2 and TGFβ_1 are also elevated in the serum of lymphoma patients (Hsu et al., 1993). We focused on IL-6 and TNFα, as both have been shown to be elevated, not only in lymphoma patients, but in many other cancer settings. Initial results showed neither IL-6 nor TNFα had a significant effect on aggregation or activation (CD62P and PAC1 binding) in response to ADP (Fig 6.10, 6.11), while aggregation and activation in response to collagen (Fig 6.12, 6.13) was significantly inhibited. However, the massive heterogeneity between samples warranted investigation into the stability of the collagen being used as an agonist. Using non-pre-diluted collagen (i.e. collagen that was not diluted in Tyrodes buffer to the required

2µg/ml prior to use) in a repeat set of experiments proved that neither IL-6 nor TNFα significantly affected aggregation or in response to collagen.

However, when the effects of the CM from the L1236 cells were retested using non-pre-diluted collagen a significant effect on both aggregation and activation (CD62P and PAC1 binding) was still observed (Fig 6.15-6.17). Therefore, other factors within the lymphoma secretome are responsible for this inhibition on platelet function.

A study by Nilsson et al. (2013) reported that platelets contain tumour-derived RNA molecules that they propose can be used in biomarker analysis. Assessment of platelet function has not been studied in this context and therefore could be the cause of the inhibition of aggregation and activation potential observed in this study following incubation with the lymphoma secretome. Dysfunctional expression of miRNAs is a common feature of malignancy (Lawrie et al., 2007).

The expression level of some miRNAs in the serum of lymphoma patients is dysregulated (Fang et al., 2012). Additionally it has been found that in HL cell lines there is a significant overexpression of miR-21, miR-15b and miR-16 and down regulation of others such as miR-150 (Gibcus et al., 2009). It is proposed that *in-vivo*, lymphoma cells may export miRNAs into the extracellular milieu, which may then travel from the lymph node to the peripheral blood to be a target for platelet uptake. Despite platelets being anucleated cells, they are capable of post-transcriptional gene regulation, owing to the messenger ribonucleic acid (mRNA) from their parent megakaryocyte during platelet production (Stakos et al., 2013; Nilsson et al., 2011; Hsu et al., 1993) Therefore, the uptake of

tumour-derived miRNA by platelets may have an effect on mRNA translation and therefore protein synthesis in the platelet (Soslau et al., 1997).

Interest in tumour-derived microparticles (MPs) has recently grown, with evidence that MPs can 'deliver' chemoresistance genes from one cancer cell to another (Martins et al., 2013). Despite their small size, MPs contain a very concentrated set of cytokines, signalling proteins, mRNA, and microRNA which originate from the cell they were shed from (Martins et al., 2013). The possibility of lymphoma-derived MPs causing changes to platelet function is not inconceivable and provides justification for studying this aspect in future work.

Collagen has been proposed to be the strongest agonist of platelet aggregation. When used to stimulate platelets in the current study, aggregation and activation levels were significant in healthy platelets. Initial data showed that incubation of the healthy platelets with either the lymphoma secretome or either one of the recombinant cytokines IL-6 or TNFα resulted in a significant inhibition of collagen-induced aggregation and activation. However, re-running untreated samples at the end of the experiments proved that decreases in aggregation were due to instability of the collagen solution during the experiment. The collagen is supplied as a stock solution of 1mg/ml but must be diluted in Tyrode's solution to a working concentration of 20μg/ml. When 50μl of this is added to a 450μl volume of PRP, this adjusts the final concentration to 2μg/ml. This is a standard protocol used by many platelet laboratories worldwide. The collagen begins to form aggregates within the Tyrode's solution and stability experiments involving repeated analysis of PRP over time showed significant loss of

effect, even when storing the solution on ice. By adding 1µl of stock collagen directly to 499µl PRP, the stability problem was resolved. Further experiments using the new method showed that collagen-induced aggregation is indeed inhibited by the lymphoma secretome, but not be either recombinant IL-6 or TNFα.

Interaction between platelets and collagen is important in the study of platelet activation, where collagen binds to platelet receptors directly or indirectly with $\alpha_2\beta_1$ and $\alpha_{IIB}\beta_3$ (via VWF). This binding is needed for high affinity confirmation in response to inside out signalling that uses GPVI receptor on platelets to provide more stable adhesion. However, different factors may influence this, such as the type of collagen in terms of preparation and species source of collagen (Nieswandt and Watson, 2003).

There are more than 20 types of collagen in humans and 9 of them have been identified to be expressed on the vessel wall, I, II, IV, V, VI, VII, VIII, XII, XIII and XIV. The major component of extracellular matrix is fibrillar type III collagen and I, which are used commonly in platelet studies. This type consists of chains that contain 1000 amino acids flanked by “short non-helical N-and C- terminal telopentide extension” (Nieswandt and Watson, 2003). This monomeric cross-linked structure forms fibrillar collagen, which needs specific preparation to use *in-vivo*, and in-*vitr*o to stimulate platelets. However, the complicated structure may lead to stability issues (Nieswandt and Watson, 2003). In this study fibril type I collagen, which is sourced from equine tendons, has been used which also has a complicated structure, as evidenced from the stability issues seen here.

In contrast to the results presented in this chapter, Soslau et al (1996) demonstrated significant stimulation of collagen-induced platelet aggregation following incubation with IL-6, while a small inhibition of aggregation was observed following incubation with TNF-α (Soslau et al., 1996). The reason for the opposing results, in terms of IL-6 between the present study and Saslow et al. remains to be fully determined but could be due to differences in post-transcriptional gene regulation within the sample population. However, no haemostatic abnormality has been reported in patients who have received IL-6 in clinical trials (Oleksowicz et al., 1994), although this direct relationship has not been the focus of any investigation.

Another study has shown significant increases in platelet aggregation when platelets were incubated with TNF-α and triggered by collagen (Pignatelli et al., 2005). This is in contrast to the results presented here. The experimental protocol did however difference between the two studies; Pignatelli et al. (2005) incubated the TNFα-treated platelets at 37^0C for 15 minutes prior to inducing aggregation by collagen, while in the present study, platelets were incubated at room temperature. P75, a platelet-specific receptor for TNF-α (Bar et al., 1997) may be more stable at 37^0C, increasing its effect on platelet function.

The data reported here are based upon low donor numbers and cannot be used to provide definitive conclusions. However, there are similarities with our work and some of the work in the literature especially when considering ADP-induced platelet aggregation following recombinant cytokine treatment (Soslau et al., 1997; Pignatelli et al., 2005). Another limitation in this study is that only two platelet agonists, collagen and

ADP have been used. Further study needs to assess whether aggregation/activation in response to alternative agonists such as Thrombin, Epinephrine and Arachidonic acid is also affected by secretome factors (Pignatelli et al., 2005).

A study by Leslie et al. (1994) has shown a significant increase in ADP-induced platelet aggregation after incubating platelets with IL-6. The disparity between this result and the results presented in the current chapter could be explained by contamination of PRP by leukocytes; IL-6 has been proposed to be involved in platelet aggregation through an alternative pathway, indirectly through mononuclear cells. These mononuclear cells express the IL-6 receptor and its activation leads to indirect activation and secretion of platelet activation agonists by the mononuclear cells themselves (Leslie et al., 1994). It is therefore imperative to ensure minimal leukocyte contamination in PRP.

It has been proposed that cytokines themselves may act as direct platelet agonists if added at the start of aggregation analysis, however, no change in platelet function was seen when this was tested (data not shown). This has been supported by Pignatelli et al (2005) who used 40pg/ml of TNF-α as the agonist for platelet aggregation and found no difference in platelet aggregation. Yet, alternative studies have reported small agonist activities for TNFα (Soslau et al., 1997). Differences in mRNA and miRNA profiles between donors used in these studies could account for differences in response, as post-transcriptional gene regulation in platelets has been shown to be extremely important in platelet behaviour. Incubation temperature and cytokine concentration also vary between these published studies (Bar et al., 1997; Pignatelli et al., 2005).

In-vitro studies of this nature present difficulties in recreating the *in-vivo* microenvironmental interactions which may be key to generating the changes in haemostatic function (Hsu et al., 1993). For example, platelets release some cytokines, which are proposed to act in concert with other cytokines released from additional cells within the microenvironment, to modulate haemostasis and thrombosis differentially dependent on the site of injury (Soslau et al., 1997). Therefore, the method used so far has its drawbacks. Whole blood aggregometry may have provided a more accurate assessment of the aggregation in this setting, to determine if the presence of other blood components exacerbates/amplifies effects seen in PRP.

In conclusion, Although the secretome of HL cell line L1236 significantly inhibits platelet aggregation and activation, direct treatment with IL-6 and TNFα which are reported to be major factors present in the secretome have no effect. Future work in this area should aim to fully characterise the factors responsible for such as strong inhibitory action on platelet function.

Chapter 7: Conclusion and future work

Chapter 7: Conclusion and future work.

7.1 Conclusion

This work tested the anti-platelet activity of six novel thienopyridine derivatives with the aim of identifying new compounds that can be taken forward for further clinical development and used as alternatives to current anti-platelet therapies. Although a number of anti-platelet therapies are currently in clinical use, the heterogeneity amongst patients in terms of response to these agents and variability in side effects warrants continued identification and refinement of alternative compounds.

The first experimental chapter demonstrated that all six novel compounds were able to inhibit ADP-stimulated platelet aggregation (assessed by LTA), platelet activation (assessed by CD62P expression, PAC-1 binding and PLT-LEUK aggregate formation), and were able to provide an additive effect when used in combination with Aspirin. Compounds DJ0199, DJ0171 and DJ0097 appeared to stand out as the most effective compounds.

The second experimental chapter demonstrated the ability of the compounds to maintain VASP phosphorylation in ADP-stimulated platelets, further proving the inhibitory action of these compounds. Again, DJ0199, DJ0171 and DJ0097 appeared to be the most effective at inhibiting platelet activity. Work also attempted to determine other signalling pathways affected by these compounds, but could not find consistent effects on cPLA2, PLCγ2 or Akt.

The third experimental chapter focused on the effects of these compounds on endothelial cells, highlighting the significance of P2Y12 inhibition on global haemostasis. The chapter demonstrated that all compounds were able to inhibit ADP-stimulated expression of CD62P, CD62E and VCAM-1 expression on HUVEC cells, but the most successful compounds appeared to be DJ0199 and DJ0171.

Taken together, the results provide justification for pursing the development of DJ0199 and DJ0171 (and possibly DJ0097) and also show that these compounds may also be used in the setting of endothelial dysfunction.

Finally, the work presented in chapter 6 focused on the immunological aspect of platelet biology and was able to show that platelet function can also be significantly affected by the tumour cell secretome. Platelet activation and aggregation was inhibited following incubation of platelets with conditioned media from Lymphoma cell lines. The results support data in the literature that describe the ability of platelets to sequester proteins from the microenvironment, and help to explain the increased bleeding tendency seen in a proportion of cancer patients.

7.2 Future work

The work focused on the use of these novel thienopyridines in platelets obtained from healthy patients. Although these platelets were stimulated with ADP following treatment with the novel compounds to mimic hyperactivity, future work should focus on obtaining platelets from patients with platelet hyperactivity. Patients with ACS, or those immediately following MI or stroke, but before being administered with a loading

dose of clopidogrel/ticagrelor would provide the ideal study setting. This would allow determination of the ability of these novel compounds to inhibit the activity of platelets with an inherently elevated baseline activation status. An additional follow on to this would be to obtain blood samples from clopidogrel-non-responders and test the efficacy of the novel derivatives. This would evaluate the benefits of these compounds in patients who cannot be treated with the mainstream treatment regime.

The work presented in this thesis worked on platelets in a static environment – i.e. the PRP was treated in tubes with the relevant compounds and incubated for 30 mins before platelet stimulation and assessment of activation/aggregation. Further work would focus on the activation and aggregation of platelets treated with these compounds under a flow system that mimics physiological blood flow. Pre-treated platelets can be perfused over a surface coated with a platelet agonists and the adhesion of platelets to the agonist-coated surface following the flow assay can be assessed by epifluorescence microscopy (Schulz et al., 2009; HEEMSKERK et al., 2011). Another useful adaptation of this assay could be a co-culture system of platelet and endothelial cells. Instead of coating a surface within the flow system with an agonist, the surface could be coated with a monolayer of HUVECs (Terrisse et al., 2010). The thienopyridine-treated platelets would then be perfused through the system, stimulated with ADP and then their adhesion to the endothelaial layer would be assessed by epifluorescence microscopy.

The pilot data presented in chapter 5, demonstrating the inhibitory effects of these compounds on endothelial cells, should be validated by analysing CD62P, CD62E, VCAM-1 and ICAM-1 in ADP-stimulated HUVECs by flow cytometry and also western blotting.

The flow cytometry would provide quantitative data that can be statistically analysed to determine the most effective novel derivative in this setting. Furthermore, as ADP has been reported to stimulate migration of HUVEC cells via the ERK1/2 pathway, analysis of the phosphorylation status of ERK1/2 kinases following thienopyridine treatment would also be useful to explore.

With regards to platelet signalling pathways affected by these compounds, western blotting proved difficult to anlyse these events that were occurring within microseconds of agonist treatment. Again, real-time flow cytometry on the Accuri C6 next generation flow cytometry would be a more effective method for assessing signal transduction in this setting. Further, expression of PLCβ would be a useful addition to the testing panel, due to its major role in Gq-coupled GPCRs including P2Y1 (alternative ADP receptor) (Szumilo and Rahden-Staron, 2008).

Neither IL-6 nor TNFα were found to significantly affect ADP-mediated or collagen-mediated activation/aggregation, despite the strong effect of the lymphoma cell secretome. Further work must characterise the factors within this secretome that are acting to inhibit platelet function. This would involve multiplex ELISA or cytometric Bead Array to characterise the exact cytokine profile of the secretome.

Chapter 8: References

Adamski, P., Adamska, U., Ostrowska, M., Navarese, E. P. and Kubica, J. J. E. o. o. p. (2018) 'Evaluating current and emerging antithrombotic therapy currently available for the treatment of acute coronary syndrome in geriatric populations.' pp. 1-11.

Aleil, B., Ravanat, C., Cazenave, J., Rochoux, G., Heitz, A. and Gachet, C. J. J. o. T. a. H. (2005) 'Flow cytometric analysis of intraplatelet VASP phosphorylation for the detection of clopidogrel resistance in patients with ischemic cardiovascular diseases.' 3(1) pp. 85-92.

AL Muller, K. and Geisler, T. J. C. v. p. (2012) 'Novel antiplatelet therapy in acute coronary syndromes: What is new in the pipeline?' 10(5) pp. 606-618.

Almog, N. and Klement, G. L. (2010) 'Platelet proteome and tumor dormancy: Can platelets content serve as predictive biomarkers for exit of tumors from dormancy?' *Cancers*

Angiolillo, D. J., Angiolillo, D. J., Capranzano, P., Desai, B. and Shoemaker, S. B. (2009) 'Impact of P2Y(12) Inhibitory Effects Induced by Clopidogrel on Platelet Procoagulant Activity in Type 2 Diabetes Mellitus Patients.' *Thrombosis research*, 124(3) pp. 318-322.

Angiolillo, D. J., Welsh, R. C., Trenk, D., Neumann, F.-J., Conley, P. B., McClure, M. W., Stephens, G., Kochman, J., Jennings, L. K. and Gurbel, P. A. J. C. C. I. (2012) 'Pharmacokinetic and pharmacodynamic effects of elinogrel: results of the platelet function substudy from the intravenous and oral administration of elinogrel to evaluate tolerability and efficacy in nonurgent percutaneous coronary intervention patients (INNOVATE-PCI) trial.' 5(3) pp. 347-356.

Antman, E. M., DeMets, D. and Loscalzo, J. J. C. (2005) 'Cyclooxygenase inhibition and cardiovascular risk.' 112(5) pp. 759-770.

Armstrong, P. C. J., Leadbeater, P. D., Chan, M. V., Kirkby, N. S., Jakubowski, J. A., Mitchell, J. A. and Warner, T. D. (2011) 'In the presence of strong P2Y12 receptor blockade, aspirin provides little additional inhibition of platelet aggregation.' *Journal of Thrombosis and Haemostasis*, 9(3) pp. 552-561.

Aslan, J. E., Itakura, A., Gertz, J. M. and McCarty, O. J. T. (2012) 'Platelet shape change and spreading.' *Methods in molecular biology (Clifton, N.J.)*, 788, 2012, pp. 91-100.

Asselin, J., Gibbins, J. M., Achison, M., Lee, Y. H., Morton, L. F., Farndale, R. W., Barnes, M. J. and Watson, S. P. (1997) 'A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase C gamma2 in platelets independent of the integrin alpha2beta1.' *Blood*, 89(4) p. 1235.

Assinger, A., Volf, I. and Schmid, D. J. P. O. (2015) 'A novel, rapid method to quantify intraplatelet Ca^{2+} dynamics by ratiometric flow cytometry.' 10(4) p. e0122527.

Bambace, N. M. and Holmes, C. E. (2011) 'The platelet contribution to cancer progression.' *Journal of Thrombosis and Haemostasis*, 9(2), Feb, pp. 237-249.

Bar, J., Zosmer, A., Hod, M., Elder, M. G. and Sullivan, M. H. F. (1997) 'The regulation of platelet aggregation in vitro by interleukin-1 beta and tumor necrosis factor-alpha: Changes in pregnancy and in pre-eclampsia.' *Thrombosis and Haemostasis*, 78(4), Oct, pp. 1255-1261.

Belt, R. J., Leite, C., Haas, C. D. and Stephens, R. L. (1978) 'INCIDENCE OF HEMORRHAGIC COMPLICATIONS IN PATIENTS WITH CANCER.' *Jama-Journal of the American Medical Association*, 239(24) pp. 2571-2574.

Benoy, I., Salgado, R., Colpaert, C., Weytjens, R., Vermeulen, P. B. and Dirix, L. Y. (2002) 'Serum Interleukin 6, Plasma VEGF, Serum VEGF, and VEGF Platelet Load in Breast Cancer Patients.' *Clinical Breast Cancer*, 2(4) pp. 311-315.

Bergmeier, W., Piffath, C. L., Goerge, T., Cifuni, S. M., Ruggeri, Z. M., Ware, J. and Wagner, D. D. (2006) 'The Role of Platelet Adhesion Receptor GPIbα Far Exceeds That of Its Main Ligand, von Willebrand Factor, in Arterial Thrombosis.' *Proceedings of the National Academy of Sciences of the United States of America*, 103(45) pp. 16900-16905.

Bhatt, D. L., Fox, K. A., Hacke, W., Berger, P. B., Black, H. R., Boden, W. E., Cacoub, P., Cohen, E. A., Creager, M. A. and Easton, J. D. J. N. E. J. o. M. (2006) 'Clopidogrel and aspirin versus aspirin alone for the prevention of atherothrombotic events.' 354(16) pp. 1706-1717.

Bhatt, D. L., Stone, G. W., Mahaffey, K. W., Gibson, C. M., Steg, P. G., Hamm, C. W., Price, M. J., Leonardi, S., Gallup, D. and Bramucci, E. J. N. E. J. o. M. (2013) 'Effect of platelet inhibition with cangrelor during PCI on ischemic events.' 368(14) pp. 1303-1313.

Bhatt, D. L. J. T. A. j. o. c. (2009) 'Role of antiplatelet therapy across the spectrum of patients with coronary artery disease.' 103(3) pp. 11A-19A.

Blair, P. and Flaumenhaft, R. (2009) 'Platelet α-granules: Basic biology and clinical correlates.' *Blood Reviews*, 23(4) pp. 177-189.

Bobe, R., Wilde, J. I., Maschberger, P., Venkateswarlu, K., Cullen, P. J., Siess, W. and Watson, S. P. (2001) 'Phosphatidylinositol 3-kinase-dependent translocation of phospholipase Cgamma2 in mouse megakaryocytes is independent of Bruton tyrosine kinase translocation.' *Blood*, 97(3) pp. 678-684.

Born, G. V. R. J. N. (1962) 'Aggregation of blood platelets by adenosine diphosphate and its reversal.' 194(4832) pp. 927-929.

Bouman, H. J., van Werkum, J. W., Rudez, G., Leebeek, F. W., Kruit, A., Hackeng, C. M., Ten Berg, J. M., de Maat, M. P. and Ruven, H. J. (2010) 'The influence of variation in the P2Y12 receptor gene on in vitro platelet inhibition with the direct P2Y12 antagonist cangrelor.' *Thromb Haemost*, 103(2), Feb, 2010/02/04, pp. 379-386.

Bouman, H. J., Schömig, E., Van Werkum, J. W., Velder, J., Hackeng, C. M., Hirschhäuser, C., Waldmann, C., Schmalz, H.-G., Ten Berg, J. M. and Taubert, D. J. N. m. (2011) 'Paraoxonase-1 is a major determinant of clopidogrel efficacy.' 17(1) p. 110.

Burch, J. W., Stanford, N. and Majerus, P. W. J. T. J. o. c. i. (1978) 'Inhibition of platelet prostaglandin synthetase by oral aspirin.' 61(2) pp. 314-319.

Bye, A. P., Unsworth, A. J. and Gibbins, J. M. (2016) 'Platelet signaling: a complex interplay between inhibitory and activatory networks.' *Journal of Thrombosis and Haemostasis*, 14(5) pp. 918-930.

Cannon, C. P., Husted, S., Harrington, R. A., Scirica, B. M., Emanuelsson, H., Peters, G., Storey, R. F. and Cardiology, D.-I. J. J. o. t. A. C. o. (2007) 'Safety, tolerability, and initial efficacy of AZD6140, the first reversible oral adenosine diphosphate receptor antagonist, compared with clopidogrel, in patients with non–ST-segment elevation acute coronary syndrome: primary results of the DISPERSE-2 trial.' 50(19) pp. 1844-1851.

Carrié, D., Garcia, C., Gratacap, M. P., Voisin, S., Payrastre, B. and Sié, P. (2009) 'Assessment of platelet response to clopidogrel through measurement of ADP-induced Akt phosphorylation.' *Journal of thrombosis and haemostasis : JTH*, 7(8) pp. 1411-1413.

Cartoni, C., Niscola, P., Breccia, M., Brunetti, G., D'Elia, G. M., Giovannini, M., Romani, C., Scaramucci, L., Tendas, A., Cupelli, L., De Fabritiis, P., Foa, R. and Mandelli, F. (2009) 'Hemorrhagic complications in patients with advanced hematological malignancies followed at home: an Italian experience.' *Leukemia & Lymphoma*, 50(3) pp. 387-391.

Caruso, V., Di Castelnuovo, A., Meschengieser, S., Lazzari, M. A., de Gaetano, G., Storti, S., Iacoviello, L. and Donati, M. B. (2010) 'Thrombotic complications in adult patients with lymphoma: a meta-analysis of 29 independent cohorts including 18 018 patients and 1149 events.' *Blood*, 115(26) p. 5322.

Cattaneo, M. and Lecchi, A. J. J. o. T. a. H. (2007) 'Inhibition of the platelet P2Y12 receptor for adenosine diphosphate potentiates the antiplatelet effect of prostacyclin.' 5(3) pp. 577-582.

Cattaneo, M. and Podda, G. M. J. I. a. e. m. (2010) 'State of the art of new P2Y 12 antagonists.' 5(5) pp. 385-391.

Cervi, D., Yip, T. T., Bhattacharya, N., Podust, V. N., Peterson, J., Abou-Slaybi, A., Naumov, G. N., Bender, E., Almog, N., Italiano, J. E., Folkman, J. and Klement, G. L. (2008)

'Platelet-associated PF-4 as a biomarker of early tumor growth.' *Blood*, 111(3), Feb, pp. 1201-1207.

Circulation, R. I. J. (1997) 'Effects of platelet glycoprotein IIb/IIIa blockade with tirofiban on adverse cardiac events in patients with unstable angina or acute myocardial infarction undergoing coronary angioplasty.' 96(5) pp. 1445-1453.

Clark, M. G. P. P. A. C. A., Beavers, C. P. A. and Osborne, J. M. D. P. F. (2015) 'Managing the acute coronary syndrome patient: Evidence based recommendations for anti-platelet therapy.' *Heart & Lung: The Journal of Acute and Critical Care*, 44(2) pp. 141-149.

Clemetson, K. J. (2011) 'Platelets and Primary Haemostasis.' *Thrombosis Research*, 129(3) pp. 220-224.

Daly, M. E., Makris, A., Reed, M. and Lewis, C. E. (2003) 'Hemostatic regulators of tumor angiogenesis: A source of antiangiogenic agents for cancer treatment?' *Journal of the National Cancer Institute*, 95(22), Nov 19, pp. 1660-1673.

Damman, P., Woudstra, P., Kuijt, W. J., de Winter, R. J. and James, S. K. (2012) 'P2Y12 platelet inhibition in clinical practice.' *Journal of Thrombosis and Thrombolysis*, 33(2), 2012/02/01, pp. 143-153.

De Caterina, R., Libby, P., Peng, H.-B., Thannickal, V. J., Rajavashisth, T., Gimbrone, M. A., Shin, W. S. and Liao, J. K. J. T. J. o. c. i. (1995) 'Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines.' 96(1) pp. 60-68

De Cristofaro, R., Landolfi, R., Di Cera, E., Castagnola, M., De Candia, E. and Bizzi, B. (1989) 'Inhibition of fibrinogen binding to human platelets by blockage of Na+/H+ exchange.' *Biochemical and biophysical research communications*, 161(3) p. 1228.

Di Minno, G., Silver, M. J. and Murphy, S. J. B. (1983) 'Monitoring the entry of new platelets into the circulation after ingestion of aspirin.' 61(6) pp. 1081-1085.

Dimechev G.A (2012) Cellular Regulation of Myoblast Migration and Mygenesis. Ph.D.

Manchester Metropolitan University

Dib, C., Hanna, E. B. and Abu-Fadel, M. S. J. T. A. j. o. t. m. s. (2010) 'A new era for antiplatelet therapy in patients with acute coronary syndrome.' 340(5) pp. 407-411.

Dorsam, R. T. and Kunapuli, S. P. (2004) 'Central role of the P2Y12 receptor in platelet activation.' *The Journal of clinical investigation*, 113(3) pp. 340-345.

Dovlatova, N., May, J. A. and Fox, S. C. (2015) 'Remote platelet function testing - Significant progress towards widespread testing in clinical practice.' *Platelets*, 26(5), Aug, pp. 399-401.

Dovlatova, N., May, J. A. and Fox, S. C. J. P. (2015) 'Remote platelet function testing–significant progress towards widespread testing in clinical practice.' 26(5) pp. 399-401.

Du, X. (2007) 'Signaling and regulation of the platelet glycoprotein Ib-IX-V complex.' *Current opinion in hematology*, 14(3) p. 262.

Emmons, K. L. and Taylor, N. R. (2007) 'Contemporary Issues in Clopidogrel Therapy: New Evidence Shaping Clinical Practice.' *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, 27(4) pp. 553-563.

Falanga, A., Marchetti, M. and Vignoli, A. (2013) 'Coagulation and cancer: biological and clinical aspects.' *Journal of Thrombosis and Haemostasis*, 11(2), Feb, pp. 223-233.

Fang, C., Zhu, D.-X., Dong, H.-J., Zhou, Z.-J., Wang, Y.-H., Liu, L., Fan, L., Miao, K.-R., Liu, P., Xu, W. and Li, J.-Y. (2012) 'Serum microRNAs are promising novel biomarkers for diffuse large B cell lymphoma.' *Ann Hematol*, 91(4) pp. 553-559.

Farid, N. A., Kurihara, A. and Wrighton, S. A. J. T. J. o. C. P. (2010) 'Metabolism and disposition of the thienopyridine antiplatelet drugs ticlopidine, clopidogrel, and prasugrel in humans.' 50(2) pp. 126-142.

Farid, N. A., McIntosh, M., Garofolo, F., Wong, E., Shwajch, A., Kennedy, M., Young, M., Sarkar, P., Kawabata, K. and Takahashi, M. J. R. C. i. M. S. A. I. J. D. t. t. R. D. o. U. t. t. M. R. i. M. S. (2007) 'Determination of the active and inactive metabolites of prasugrel in human plasma by liquid chromatography/tandem mass spectrometry.' 21(2) pp. 169-179.

Fontana, P. and Reny, J.-L. (2008) 'Frequently asked questions on clopidogrel treatment: indications, resistance, and biological evaluation in vascular patients.' *Revue medicale suisse*, 4(143) p. 360.

Franchi, F. and Angiolillo, D. J. (2015) 'Novel antiplatelet agents in acute coronary syndrome.' *Nature reviews. Cardiology*, 12(1) pp. 30-47.

Franchi, F., Rollini, F., Cho, J. R., Ferrante, E. and Angiolillo, D. J. J. C. t. o. i. c. m. (2014) 'Platelet function testing in contemporary clinical and interventional practice.' 16(5) p. 300.

Franchini, M., Favaloro, E. J. and Lippi, G. J. C. C. A. (2010) 'Glanzmann thrombasthenia: an update.' 411(1-2) pp. 1-6.

Freedman, J. E. (2003) 'CD40-CD40L and platelet function - Beyond hemostasis.' *Circulation Research*, 92(9), May, pp. 944-946.

Frere, C., Cuisset, T., Quilici, J., Camoin, L., Carvajal, J., Morange, P. E., Lambert, M., Juhan-Vague, I., Bonnet, J.-L. and Alessi, M.-C. (2007) 'ADP-induced platelet aggregation and platelet reactivity index VASP are good predictive markers for clinical outcomes in non-ST elevation acute coronary syndrome.' *Thrombosis and Haemostasis*, 98(4) pp. 838-843.

Fu, B. H., Fu, Z. Z., Meng, W., Gu, T., Sun, X. D. and Zhang, Z. (2015) 'Platelet VEGF and serum TGF-beta 1 levels predict chemotherapy response in non-small cell lung cancer patients.' *Tumor Biology*, 36(8), Aug, pp. 6477-6483.

Furman, M. I., Benoit, S. E., Barnard, M. R., Valeri, C. R., Borbone, M. L., Becker, R. C., Hechtman, H. B. and Michelson, A. D. (1998) 'Increased Platelet Reactivity and Circulating Monocyte-Platelet Aggregates in Patients With Stable Coronary Artery Disease.' *Journal of the American College of Cardiology*, 31(2) pp. 352-358.

Furman, M. I., Barnard, M. R., Krueger, L. A., Fox, M. L., Shilale, E. A., Lessard, D. M., Marchese, P., Frelinger, r. A. L., Goldberg, R. J. and Michelson, A. D. (2001) 'Circulating monocyte-platelet aggregates are an early marker of acute myocardial infarction.' *Journal of the American College of Cardiology*, 38(4) pp. 1002-1006.

Gachet, C. (2006) 'Regulation of platelet functions by P2 receptors.' *Annual review of pharmacology and toxicology*, 46(1) pp. 277-300.

Gachet, C. (2008) 'P2 receptors, platelet function and pharmacological implications.' *Thrombosis and Haemostasis*, 99(3) pp. 466-472.

Garcia, A., Shankar, H., Murugappan, S., Kim, S. and Kunapuli, S. P. (2007) 'Regulation and functional consequences of ADP receptor-mediated ERK2 activation in platelets.' *The Biochemical journal*, 404(2) pp. 299-308.

Giachini, F. R., Leite, R., Osmond, D. A., Lima, V. V., Inscho, E. W., Webb, R. C. and Tostes, R. C. J. P. o. (2014) 'Anti-platelet therapy with clopidogrel prevents endothelial dysfunction and vascular remodeling in aortas from hypertensive rats.' 9(3) p. e91890.

Gibbins, J., Mahaut-Smith, M. P. and Gibbins, J. M. (2004) *Platelets and Megakaryocytes, Volume 2 : Perspectives and Techniques.* Vol. 273. Totowa: Humana Press.

Gibbins, J. M. and Mahaut-Smith, M. P. (2012) *Platelets and Megakaryocytes: Volume 3, Additional Protocols and Perspectives.* Springer.

Gibcus, J. H., Tan, L. P., Harms, G., Schakel, R. N., de Jong, D., Blokzijl, T., Möller, P., Poppema, S., Kroesen, B.-J. and van Den Berg, A. (2009) 'Hodgkin Lymphoma Cell Lines Are Characterized by a Specific miRNA Expression Profile.' *Neoplasia*, 11(2) pp. 167,IN166-176,IN169.

Gilio, K., Munnix, I. C. A., Mangin, P., Cosemans, J. M. E. M., Feijge, M. A. H., van der Meijden, P. E. J., Olieslagers, S., Chrzanowska-Wodnicka, M. B., Lillian, R., Schoenwaelder, S., Koyasu, S., Sage, S. O., Jackson, S. P. and Heemskerk, J. W. M. (2009) 'Non-redundant roles of phosphoinositide 3-kinase isoforms alpha and beta in glycoprotein VI-induced platelet signaling and thrombus formation.' *The Journal of biological chemistry*, 284(49) pp. 33750-33762.

Glenn, J. R., White, A. E., Johnson, A., Fox, S. C., Behan, M. W., Dolan, G. and Heptinstall, S. (2005) 'Leukocyte count and leukocyte ecto-nucleotidase are major determinants of the effects of adenosine triphosphate and adenosine diphosphate on platelet aggregation in human blood.' *Platelets*, 16(3-4), May-Jun, 2005/07/14, pp. 159-170.

Goff, J. D. C., Lloyd-Jones, D. M., Bennett, G., Coady, S., D'Agostino, R. B., Gibbons, R., Greenland, P., Lackland, D. T., Levy, D., O'Donnell, C. J., Robinson, J. G., Schwartz, J. S., Shero, S. T., Smith, J. S. C., Sorlie, P., Stone, N. J. and Wilson, P. W. F. (2014) '2013 ACC/AHA Guideline on the Assessment of Cardiovascular Risk: A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines.' *Circulation*, 129(25_suppl_2 Suppl 1) pp. S49-S73.

Goldschmidt, N., Linetsky, E., Shalom, E., Varon, D. and Siegal, T. (2003) 'High incidence of thromboembolism in patients with central nervous system lymphoma.' *Cancer*, 98(6), Sep, pp. 1239-1242.

Goldsmith, H. L. and Turitto, V. T. (1986) 'Rheological aspects of thrombosis and haemostasis: basic principles and applications. ICTH-Report--Subcommittee on Rheology of the International Committee on Thrombosis and Haemostasis.' *Thrombosis and haemostasis*, 55(3) p. 415.

Granger, D. N. and Senchenkova, E. (2010) *Inflammation and the Microcirculation.* Vol. 2: Morgan & Claypool Life Sciences.

Gräbner, R., Till, U. and Heller, R. J. C. T. J. o. t. I. S. f. A. C. (2000) 'Flow cytometric determination of E-selectin, vascular cell adhesion molecule-1, and intercellular cell adhesion molecule-1 in formaldehyde-fixed endothelial cell monolayers.' 40(3) pp. 238-244.

Gremmel, T., Gremmel, T. and Panzer, S. (2011) 'Clinical, genetic and confounding factors determine the dynamics of the in vitro response/non response to clopidogrel.' *Thrombosis and haemostasis*, 106(2) pp. 211-218.

Gremmel, T., Eslam, R. B., Koppensteiner, R., Lang, I. M. and Panzer, S. (2013) 'Prasugrel Reduces Agonists' Inducible Platelet Activation and Leukocyte–Platelet Interaction more efficiently than Clopidogrel.' *Cardiovascular Therapeutics*, 31(5) pp. e40-e45.

Gresele, P., Gustav, V., Patrono, C. and Page, C. P. (2012) *Antiplatelet agents.* Vol. 210. Springer Science & Business Media.

Group, T. S. C. J. T. L. (1991) 'Swedish Aspirin Low-dose Trial (SALT) of 75 mg aspirin as secondary prophylaxis after cerebrovascular ischaemic events.' 338(8779) pp. 1345-1349.

Gurbel, P. A. and Tantry, U. S. J. C. (2012) 'Platelet Function Testing and Genotyping Improve Outcome in Patients Treated With Antithrombotic AgentsResponse to Gurbel and Tantry.' 125(10) pp. 1276-1287.

Gurbel, P. A., Bliden, K. P., Butler, K., Tantry, U. S., Gesheff, T., Wei, C., Teng, R., Antonino, M. J., Patil, S. B. and Karunakaran, A. J. C. (2009) 'Randomized double-blind assessment of the ONSET and OFFSET of the antiplatelet effects of ticagrelor versus clopidogrel in patients with stable coronary artery disease: the ONSET/OFFSET study.' 120(25) pp. 2577-2585.

Leinco Technologies, (2018) General Western Blot Protocol. [online][Accessed on 3rd October 2018] https://www.leinco.com/western-blotting-protocol.

Harding, S. A., Sarma, J., Din, J. N., Maciocia, P. M., Newby, D. E. and Fox, K. A. A. (2006) 'Clopidogrel reduces platelet-leucocyte aggregation, monocyte activation and RANTES secretion in type 2 diabetes mellitus.' *Heart (British Cardiac Society)*

Harper, M. T. and Poole, A. W. (2010) 'Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation.' *Journal of Thrombosis and Haemostasis*, 8(3) pp. 454-462.

Harrington, R. A., Stone, G. W., McNulty, S., White, H. D., Lincoff, A. M., Gibson, C. M., Pollack, J. C. V., Montalescot, G., Mahaffey, K. W., Kleiman, N. S., Goodman, S. G., Amine, M., Angiolillo, D. J., Becker, R. C., Chew, D. P., French, W. J., Leisch, F., Parikh, K. H., Skerjanec, S. and Bhatt, D. L. (2009) 'Platelet inhibition with cangrelor in patients undergoing PCI.' *The New England journal of medicine*

Hato, T., Pampori, N. and Shattil, S. J. (1998) 'Complementary roles for receptor clustering and conformational change in the adhesive and signaling functions of integrin alphaIIb beta3.' *The Journal of cell biology*, 141(7) p. 1685.

Hechler, B., Cattaneo, M. and Gachet, C. (2005) *The P2 receptors in platelet function.* Vol. 31: Copyright© 2005 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA.

HEEMSKERK, J. W. M., SAKARIASSEN, K. S., ZWAGINGA, J. J., BRASS, L. F., JACKSON, S. P. and FARNDALE, R. W. (2011) 'Collagen surfaces to measure thrombus formation under flow: possibilities for standardization.' *Journal of Thrombosis and Haemostasis*, 9(4) pp. 856-858.

Heim, C., Gebhardt, J., Ramsperger-Gleixner, M., Jacobi, J., Weyand, M. and Ensminger, S. M. J. H. a. v. (2016) 'Clopidogrel significantly lowers the development of atherosclerosis in ApoE-deficient mice in vivo.' 31(5) pp. 783-794.

Hess, C. N., Kou, R., Johnson, R. P., Li, G. K. and Michel, T. J. J. o. B. C. (2009) 'ADP Signaling in Vascular Endothelial Cells ADP-DEPENDENT ACTIVATION OF THE ENDOTHELIAL ISOFORM OF NITRIC-OXIDE SYNTHASE REQUIRES THE EXPRESSION BUT NOT THE KINASE ACTIVITY OF AMP-ACTIVATED PROTEIN KINASE.' 284(47) pp. 32209-32224.

Hezard, N., Metz, D., Garnotel, R., Simon, G., Mace, C., Koebel, P. and Nguyen, P. (2005) 'Platelet VASP phosphorylation assessment in clopidogrel-treated patients: Lack of agreement between Western blot and flow cytometry.' *Platelets*, 16(8) pp. 474-481.

Hjemdahl, P., Chronos, N. A., Wilson, D. J., Bouloux, P. and Goodall, A. H. (1994) 'Epinephrine sensitizes human platelets in vivo and in vitro as studied by fibrinogen binding and P-selectin expression.' *Arteriosclerosis and thrombosis : a journal of vascular biology*, 14(1) p. 77.

Hochholzer, W., Trenk, D., Frundi, D., Blanke, P., Fischer, B., Andris, K., Bestehorn, H.-P., Büttner, H. J. and Neumann, F.-J. J. C. (2005) 'Time dependence of platelet inhibition after a 600-mg loading dose of clopidogrel in a large, unselected cohort of candidates for percutaneous coronary intervention.' 111(20) pp. 2560-2564.

Hoffbrand, A. V. (2011) *Postgraduate haematology.* Vol. 6th. Chichester: Wiley-Blackwell.

Hoffbrand, A. V., Moss, P. A. H. and Pettit, J. E. (2006) *Essential haematology.* Vol. 5th. Malden, Mass;Oxford;: Blackwell.

Hoffbrand, A. V., Moss, P. A. H. and Pettit, J. E. (2011) *Essential haematology.* Malden, Mass: Wiley-Blackwell.

Howard, G., Wagenknecht, L. E., Burke, G. L., Diez-Roux, A., Evans, G. W., McGovern, P., Nieto, F. J. and Tell, G. S. (1998) 'Cigarette smoking and progression of atherosclerosis: The Atherosclerosis Risk in Communities (ARIC) Study.' *JAMA*, 279(2) p. 119.

Hsu, S. M., Waldron, J. W., Hsu, P. L. and Hough, A. J. (1993) 'CYTOKINES IN MALIGNANT-LYMPHOMAS - REVIEW AND PROSPECTIVE EVALUATION.' *Human Pathology*, 24(10), Oct, pp. 1040-1057.

Hu, H. and Hoylaerts, M. F. (2010) 'The P2X1 ion channel in platelet function.' *Platelets*, 21(3) 2010/03/06, pp. 153-166.

Huo, Y., Schober, A., Forlow, S. B., Smith, D. F., Hyman, M. C., Jung, S., Littman, D. R., Weber, C. and Ley, K. J. N. m. (2003) 'Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E.' 9(1) p. 61.

Hulot, J. S., Bura, A., Villard, E. and Azizi, M. (2006) 'Cytochrorne P4502C19 loss-of-function polymorphism is a major determinant of clopidogrel responsiveness in healthy subjects.' *Blood*, 108(7) pp. 2244-2247.

Ibanez, B., James, S., Agewall, S., Antunes, M. J., Bucciarelli-Ducci, C., Bueno, H., Caforio, A. L. P., Crea, F., Goudevenos, J. A., Halvorsen, S., Hindricks, G., Kastrati, A., Lenzen, M. J., Prescott, E., Roffi, M., Valgimigli, M., Varenhorst, C., Vranckx, P. and Widimsky, P. (2018) '2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: The Task Force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology (ESC).' *Eur Heart J*, 39(2), Jan 7, 2017/09/10, pp. 119-177.

ISIS, I. J. L. 'collaboration group (1988) Randomised trial of intravenous streptokinase, oral aspirin, both, or neither, among 17187 cases of suspected acute myocardial infarction: ISIS II.' 2 pp. 349-360.

Italiano, J. E., Richardson, J. L., Patel-Hett, S., Battinelli, E., Zaslavsky, A., Short, S., Ryeom, S., Folkman, J. and Klement, G. L. (2008) 'Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released.' *Blood*, 111(3), Feb, pp. 1227-1233.

Jain, N., Li, X., Adams-Huet, B., Sarode, R., Toto, R. D., Banerjee, S. and Hedayati, S. S. (2016) 'Differences in Whole Blood Platelet Aggregation at Baseline and in Response to Aspirin and Aspirin Plus Clopidogrel in Patients with vs. without Chronic Kidney Disease.' *The American journal of cardiology*, 117(4), 12/07, pp. 656-663.

Jia, M., Li, Z., Chu, H., Li, L. and Chen, K. (2015) 'Novel oral P2Y12 inhibitor prasugrel vs. clopidogrel in patients with acute coronary syndrome: evidence based on 6 studies.' *Medical science monitor : international medical journal of experimental and clinical research*

Johan, W. M. H., Bevers, E. M. and Lindhout, T. (2002) 'Platelet Activation and Blood Coagulation.' *Thrombosis and Haemostasis*, 88(2) pp. 186-193.

Joo, S. J. (2012) 'Mechanisms of Platelet Activation and Integrin alpha II beta 3.' *KOREAN CIRCULATION JOURNAL*, 42(5) pp. 295-301.

Kahn, M. L., Zheng, Y. W., Huang, W., Bigornia, V., Zeng, D., Moff, S., Farese, J. R. V., Tam, C. and Coughlin, S. R. (1998) 'A dual thrombin receptor system for platelet activation.' *Nature*, 394(6694) p. 690.

Kannan, M. and Saxena, R. J. C. a. A. T. H. (2009) 'Glanzmann's thrombasthenia: an overview.' 15(2) pp. 152-165.

Kerr, B. A., Miocinovic, R., Smith, A. K., Klein, E. A. and Byzova, T. V. (2010) 'Comparison of Tumor and Microenvironment Secretomes in Plasma and in Platelets during Prostate Cancer Growth in a Xenograft Model.' *Neoplasia*, 12(5), May, pp. 388-U384.

Khan, B. V., Harrison, D. G., Olbrych, M. T., Alexander, R. W. and Medford, R. M. J. P. o. t. N. A. o. S. (1996) 'Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells.' 93(17) pp. 9114-9119.

Kim, M. H., Moo Hyun, K., Long Zhe, G., Eun-Seok, S. and Soe Hee, A. (2016) 'RELEVANCE OF THE CYP2C19 POLYMORPHISM FOR LOADING AND MAINTENANCE DOSE OF PRASUGREL AND CLOPIDOGREL TREATMENT EFFECT IN CORONARY ARTERY DISEASE PATIENTS UNDERGOING PERCUTANEOUS CORONARY INTERVENTION PCI : THE PRAISE-GENE STUDY.' *Journal of the American College of Cardiology*, 67(13)

Kim, S., Jin, J. and Kunapuli, S. P. (2004) 'Akt Activation in Platelets Depends on Gi Signaling Pathways.' *Journal of Biological Chemistry*, 279(6) pp. 4186-4195.

Klasters.J, Daneau, D. and Verhest, A. (1972) 'CAUSES OF DEATH IN PATIENTS WITH CANCER.' *European Journal of Cancer*, 8(2) pp. 149-&.

Klement, G., Kikuchi, L., Kieran, M., Almog, N., Yip, T. T. and Folkman, J. (2004) 'Early tumor detection using platelet uptake of angiogenesis regulators.' *Blood*, 104(11), Nov, pp. 239A-240A.

Klement, G. L., Yip, T. T., Cassiola, F., Kikuchi, L., Cervi, D., Podust, V., Italiano, J. E., Wheatley, E., Abou-Slaybi, A., Bender, E., Almog, N., Kieran, M. W. and Folkman, J. (2009) 'Platelets actively sequester angiogenesis regulators.' *Blood*, 113(12), Mar, pp. 2835-2842.

Klinkhardt, U. and Harder, S. (2005) *Flow cytometric measurement of platelet-leukocyte aggregates: a possible target to monitor platelet function?* Vol. 31: GEORG THIEME VERLAG.

Klinkhardt, U., Bauersachs, R., Adams, J., Graff, J., Lindhoff-Last, E. and Harder, S. (2003) 'Clopidogrel but not aspirin reduces P-selectin expression and formation of platelet-leukocyte aggregates in patients with atherosclerotic vascular disease.' *Clinical pharmacology and therapeutics*, 73(3) p. 232.

Kowalczyk, M., Banach, M., Mikhailidis, D. P., Hannam, S. and Rysz, J. J. M. S. M. (2009) 'Ticagrelor--a new platelet aggregation inhibitor in patients with acute coronary syndromes. An improvement of other inhibitors?' 15(12) pp. MS24-MS30.

Kroner, C., Eybrechts, K. and Akkerman, J.-W. N. (2000) 'Dual Regulation of Platelet Protein Kinase B.' *Journal of Biological Chemistry*, 275(36) pp. 27790-27798.

Kuckleburg, C. J., Yates, C. M., Kalia, N., Zhao, Y., Nash, G. B., Watson, S. P. and Rainger, G. E. J. C. r. (2011) 'Endothelial cell-borne platelet bridges selectively recruit monocytes in human and mouse models of vascular inflammation.' 91(1) pp. 134-141.

Kunapuli, S. P., Dorsam, R. T., Kim, S. and Quinton, T. M. (2003) 'Platelet purinergic receptors.' *Current Opinion in Pharmacology*, 3(2) pp. 175-180.

Laine, M., Paganelli, F. and Bonello, L. (2016) 'P2Y12-ADP receptor antagonists: Days of future and past.' *World Journal of Cardiology*, 8(5), 05/26 05/04/received 12/23/revised01/21/accepted, pp. 327-332.

Lancet, C. I. J. (1997) 'Randomised placebo-controlled trial of abciximab before and during coronary intervention in refractory unstable angina: the CAPTURE study.' 349 pp. 1429-1435.

Lancet, C. S. C. J. T. (1996) 'A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE).' 348(9038) pp. 1329-1339.

Lancet, E. I. J. (2000) 'Novel dosing regimen of eptifibatide in planned coronary stent implantation (ESPRIT): a randomised, placebo-controlled trial.' 356(9247) p. 2037.

Lancet, R. g. J. T. (1990) 'Risk of myocardial infarction and death during treatment with low dose aspirin and intravenous heparin in men with unstable coronary artery disease.' 336(8719) pp. 827-830.

Lawrie, C. H., Soneji, S., Marafioti, T., Cooper, C. D. O., Palazzo, S., Paterson, J. C., Cattan, H., Enver, T., Mager, R., Boultwood, J., Wainscoat, J. S. and Hatton, C. S. R. (2007) 'Microrna expression distinguishes between germinal center B cell- like and activated B cell- like subtypes of diffuse large B cell lymphoma.' *International Journal of Cancer*, 121(5) pp. 1156-1161.

Lawson, C. and Wolf, S. J. P. R. (2009) 'ICAM-1 signaling in endothelial cells.' 61(1) pp. 22-32.

Ley, K., Miller, Y. I. and Hedrick, C. C. J. A., thrombosis, and vascular biology. (2011) 'Monocyte and macrophage dynamics during atherogenesis.' 31(7) pp. 1506-1516.

Liao, J. K. J. C. c. (1998) 'Endothelium and acute coronary syndromes.' 44(8) pp. 1799-1808.

Li, Z., Wang, Y., Zhao, X., Liu, L., Wang, D., Wang, C., Meng, X., Li, H., Pan, Y., Wang, X., Yang, X., Zhang, C., Jing, J., Xian, Y. and Johnston, S. C. (2016) 'Treatment Effect of Clopidogrel Plus Aspirin Within 12 Hours of Acute Minor Stroke or Transient Ischemic Attack.' *J Am Heart Assoc*, 5(3), Mar 21, 2016/03/24, p. e003038.

Li, Z. Y., Delaney, M. K., O'Brien, K. A. and Du, X. P. (2010) 'Signaling During Platelet Adhesion and Activation.' *Arteriosclerosis Thrombosis and Vascular Biology*, 30(12), Dec, pp. 2341-2349.

Loll, P. J., Picot, D. and Garavito, R. M. J. N. S. a. M. B. (1995) 'The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H2 synthase.' 2(8) p. 637.

Lopes-Virella, M. F. and Virella, G. J. D. (1992) 'Immune mechanisms of atherosclerosis in diabetes mellitus.' 41(Supplement 2) pp. 86-91.

Lukasik, M., Rozalski, M., Luzak, B., Michalak, S., Kozubski, W. and Watala, C. (2010) 'Platelet activation and reactivity in the convalescent phase of ischaemic stroke.' *Thrombosis and Haemostasis*, 103(3), Mar, pp. 644-650.

Macías, C., Villaescusa, R., del Valle, L., Boffil, V., Cordero, G., Hernández, A., Hernández, P. and Ballester, J. M. J. R. e. d. c. (2003) 'Endothelial adhesion molecules ICAM-1, VCAM-1 and E-selectin in patients with acute coronary syndrome.' 56(02) pp. 137-144.

Mallouk, N., Varvat, J., Berger, A., Epinat, M., Accassat, S., Garcin, A., Montmartin, A., Li, G., Garnier, P., Mismetti, P. and Lambert, C. (2018) 'Assessment of a flow cytometry technique for studying signaling pathways in platelets: Monitoring of VASP phosphorylation in clinical samples.' *Practical Laboratory Medicine*, 11 pp. 10-18.

Mangin, P., Nonne, C., Eckly, A., Ohlmann, P., Freund, M., Nieswandt, B., Cazenave, J. P., Gachet, C. and Lanza, F. (2003) 'A PLCγ2-independent platelet collagen aggregation requiring functional association of GPVI and integrin α2β1.' *FEBS Letters*, 542(1-3) pp. 53-59.

Martins, V. R., Dias, M. S. and Hainaut, P. (2013) 'Tumor-cell-derived microvesicles as carriers of molecular information in cancer.' *Current Opinion in Oncology*, 25(1), Jan, pp. 66-75.

Massarelli, L., Musumeci, G., Bussolino, C. and Tomaselli, V. J. C. M. I. (2011) 'Diabetes mellitus and clopidogrel “resistance”.' 5(2S) pp. 55-58.

May, J. A., Heptinstall, S., Gibbins, J. M. and Mahaut-Smith, M. P. (2004) 'Platelets and Megakaryocytes: Volume 1: Functional Assays.'

McFadyen, J. D., Schaff, M. and Peter, K. J. N. R. C. (2018) 'Current and future antiplatelet therapies: Emphasis on preserving haemostasis.' 15(3) p. 181.

McKenzie, S. B. (1996) *Textbook of hematology.* Vol. 2nd. Baltimore;London;: Williams & Wilkins.

Medicine, C. i. (2001) 'Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation.' 345(7) pp. 494-502.

Medicine, E. I. (1994) 'Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty.' 330(14) pp. 956-961.

Medicine, E. I. (1997) 'Platelet glycoprotein IIb/IIIa receptor blockade and low-dose heparin during percutaneous coronary revascularization.' 336(24) pp. 1689-1697.

Medicine, P. T. (1998) 'Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes.' 339(7) pp. 436-443.

Mehta, S. R., Yusuf, S., Peters, R. J., Bertrand, M. E., Lewis, B. S., Natarajan, M. K., Malmberg, K., Rupprecht, H.-J., Zhao, F. and Chrolavicius, S. J. T. L. (2001) 'Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study.' 358(9281) pp. 527-533.

Mehta, S. R. J. N. E. J. O. M. (2010) 'Dose Comparisons of Clopidogrel and Aspirin in Acute Coronary Syndromes (vol 363, pg 930, 2010).' 363(16) pp. 1585-1585.

Michelson, A. D. (2013) *Platelets.*

Michelson, A. D., Frelinger, A. L. and Furman, M. I. (2006) 'Current options in platelet function testing.' *American Journal of Cardiology*, 98(10), Nov, pp. 4N-10N.

Michelson, A. D., Frelinger III, A. L. and Furman, M. I. J. T. A. j. o. c. (2006) 'Current options in platelet function testing.' 98(10) pp. S4-S10.

Michelson, A. D., Barnard, M. R., Krueger, L. A., Valeri, C. R. and Furman, M. I. (2001) 'Circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin: studies in baboons, human coronary

intervention, and human acute myocardial infarction.' *Circulation*, 104(13) pp. 1533-1537.

Michelson, A. D., Frelinger III, A. L., Braunwald, E., Downey, W. E., Angiolillo, D. J., Xenopoulos, N. P., Jakubowski, J. A., Li, Y., Murphy, S. A. and Qin, J. J. E. h. j. (2009) 'Pharmacodynamic assessment of platelet inhibition by prasugrel vs. clopidogrel in the TRITON-TIMI 38 trial.' 30(14) pp. 1753-1763.

Michelson, A. D. J. C. (2004) 'Platelet function testing in cardiovascular diseases.' 110(19) pp. e489-e493.

Moore, S. F., van den Bosch, M. T. J., Hunter, R. W., Sakamoto, K., Poole, A. W. and Hers, I. (2013) 'Dual regulation of glycogen synthase kinase 3 (GSK3)α/β by protein kinase C (PKC)α and Akt promotes thrombin-mediated integrin αIIbβ3 activation and granule secretion in platelets.' *The Journal of biological chemistry*, 288(6) pp. 3918-3928.

Morgan, K. G. J. B. j. (1993) ' Ca^{2+} i versus [Ca^{2+}] i.' 65(2) p. 561.

Morrell, C. N., Aggrey, A. A., Chapman, L. M. and Modjeski, K. L. (2014) 'Emerging roles for platelets as immune and inflammatory cells.' *Blood*, 123(18), May, pp. 2759-2767.

Moshfegh, K., Redondo, M., Julmy, F., Wuillemin, W. A., Gebauer, M. U., Haeberli, A. and Meyer, B. J. (2000) 'Antiplatelet effects of clopidogrel compared with aspirin after myocardial infarction: enhanced inhibitory effects of combination therapy.' *Journal of the American College of Cardiology*, 36(3) pp. 699-705.

Mousa, S. A., Bozarth, J. M., Lorelli, W., Forsythe, M. S., Thoolen, M. J., Slee, A. M., Reilly, T. M. and Friedman, P. A. J. J. o. P. a. E. T. (1998) 'Antiplatelet efficacy of XV459, a novel nonpeptide platelet GPIIb/IIIa antagonist: comparative platelet binding profiles with c7E3.' 286(3) pp. 1277-1284.

Müller, K. A. L., Geisler, T., & Gawaz, M. (2012). Elinogrel, an orally and intravenously available ADP-receptor antagonist. *Hämostaseologie*, *32*(03), 191-194.

Murugappa, S. and Kunapuli, S. P. J. F. B. (2006) 'The role of ADP receptors in platelet function.' 11 pp. 1977-1986.

Murugappa, S. and Kunapuli, S. P. (2006) 'The role of ADP receptors in platelet function.' *Front Biosci*, 11, May 1, 2005/12/22, pp. 1977-1986.

Nieswandt, B. and Watson, S. P. (2003) 'Platelet-collagen interaction: is GPVI the central receptor?' *Blood*, 102(2), Jul 15, pp. 449-461.

Nikolaus, S. and Robert, A. B. a. D. S. (2012) 'Clinical Use of Clopidogrel.' *Current Pharmaceutical Design*, 18(33) pp. 5224-5239.

Nilsson, R. J. A., Balaj, L., Hulleman, E., van Rijn, S., Pegtel, D. M., Walraven, M., Widmark, A., Gerritsen, W. R., Verheul, H. M., Vandertop, W. P., Noske, D. P., Skog, J. and Würdinger, T. (2011) 'Blood platelets contain tumor- derived RNA biomarkers.' *Blood*, 118(13) p. 3680.

Nishikawa, M., Tanaka, T. and Hidaka, H. (1980) ' Ca^{2+}-calmodulin-dependent phosphorylation and platelet secretion.' *Nature*, 287(5785) p. 863.

Nofer, J. R., Brodde, M. F. and Kehrel, B. E. J. C. a. E. P. a. P. (2010) 'High-density lipoproteins, platelets and the pathogenesis of atherosclerosis.' 37(7) pp. 726-735.

Nonne, C., Lenain, N., Hechler, B., Mangin, P., Cazenave, J.-P., Gachet, C. and Lanza, F. (2005) 'Importance of platelet phospholipase Cgamma2 signaling in arterial thrombosis as a function of lesion severity.' *Arteriosclerosis, thrombosis, and vascular biology*, 25(6) p. 1293.

Nurden, P., Nurden, A., Jantzen, H.-M., England, L., Li, G., Julius, D., Conley, P. B., Hollopeter, G., Vincent, D., Ramakrishnan, V. and Yang, R.-B. (2001) 'Identification of the platelet ADP receptor targeted by antithrombotic drugs.' *Nature*, 409(6817) pp. 202-207.

O'brien, J. J. J. o. c. p. (1962) 'Platelet aggregation: Part I Some effects of the adenosine phosphates, thrombin, and cocaine upon platelet adhesiveness.' 15(5) pp. 446-452.

Offermanns, S., Laugwitz, K.-L., Spicher, K. and Schultz, G. (1994) 'G proteins of the G(12) family are activated via thromboxane A(2) and thrombin receptors in human platelets.' *Proceedings of the National Academy of Sciences of the United States of America*, 91(2) p. 504.

Oh, P. C., Ahn, T., Kim, D. W., Hong, B. K., Kim, D. S., Kwan, J., Choi, C. U., Yang, Y. M., Bae, J. H., Jung, K. T., Choi, W. G., Jeon, D. W., Cho, D. K., Pyun, W. B., Cha, K. S., Cha, T. J., Chun, K. J., Kim, Y. D., Kim, B. S., Kim, D. I. and Kim, T. I. (2016) 'Comparative effect on platelet function of a fixed-dose aspirin and clopidogrel combination versus separate formulations in patients with coronary artery disease: A phase IV, multicenter, prospective, 4-week non-inferiority trial.' *Int J Cardiol*, 202, Jan 1, 2015/10/04, pp. 331-335.

Oleksowicz, L., Mrowiec, Z., Zuckerman, D., Isaacs, R., Dutcher, J. and Puszkin, E. (1994) 'PLATELET ACTIVATION-INDUCED BY INTERLEUKIN-6 - EVIDENCE FOR A MECHANISM INVOLVING ARACHIDONIC-ACID METABOLISM.' *Thrombosis and Haemostasis*, 72(2), Aug, pp. 302-308.

Ott, I., Neumann, F. J., Gawaz, M., Schmitt, M. and Schömig, A. (1996) 'Increased neutrophil-platelet adhesion in patients with unstable angina.' *Circulation*, 94(6) pp. 1239-1246.

Pampuch, A., Cerletti, C. and Gaetano, G. d. (2006) 'Comparison of VASP-phosphorylation assay to light-transmission aggregometry in assessing inhibition of the platelet ADP P2Y12 receptor.' *Thrombosis and Haemostasis*, 96(6) pp. 767-773.

Patrono, C., Coller, B., FitzGerald, G. A., Hirsh, J. and Roth, G. J. C. (2004) 'Platelet-active drugs: the relationships among dose, effectiveness, and side effects: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy.' 126(3) pp. 234S-264S.

, C. J. E. h. j. (2013) 'Low-dose aspirin in primary prevention: cardioprotection, chemoprevention, both, or neither?' 34(44) pp. 3403-3411.

Payrastre, B., Missy, K., Trumel, C., Bodin, S., Plantavid, M. and Chap, H. J. B. p. (2000) 'The integrin αIIb/β3 in human Alan MichelsonAlan MichelsonAlan Michelson signal transduction.' 60(8) pp. 1069-1074.

Peterson, J. E., Zurakowski, D., Italiano, J. E., Michel, L. V., Connors, S., Oenick, M., D'Amato, R. J., Klement, G. L. and Folkman, J. (2012) 'VEGF, PF4 and PDGF are elevated in platelets of colorectal cancer patients.' *Angiogenesis*, 15(2), Jun, pp. 265-273.

Phillips, D. R. and Scarborough, R. M. (1997) 'Clinical Pharmacology of Eptifibatide.' *The American Journal of Cardiology*, 80(4) pp. 11B-20B.

Pignatelli, P., De Biase, L., Lenti, L., Tocci, G., Brunelli, A., Cangemi, R., Riondino, S., Grego, S., Volpe, M. and Violi, F. (2005) 'Tumor necrosis factor-alpha as trigger of platelet activation in patients with heart failure.' *Blood*, 106(6) pp. 2005-2003-1247v2001-1994.

Poole, A. W., Pula, G., Hers, I., Crosby, D. and Jones, M. L. (2004) 'PKC-interacting proteins: from function to pharmacology.' *Trends in Pharmacological Sciences*, 25(10) pp. 528-535.

Postadzhiyan, A. S., Tzontcheva, A. V., Kehayov, I. and Finkov, B. J. C. b. (2008) 'Circulating soluble adhesion molecules ICAM-1 and VCAM-1 and their association with clinical outcome, troponin T and C-reactive protein in patients with acute coronary syndromes.' 41(3) pp. 126-133.

Puri, R. N. (1998) 'Phospholipase A2: Its role in ADP- and thrombin-induced platelet activation mechanisms.' *International Journal of Biochemistry and Cell Biology*, 30(10) pp. 1107-1122.

Puzon-McLaughlin, W., Kamata, T. and Takada, Y. J. J. o. B. C. (2000) 'Multiple discontinuous ligand-mimetic antibody binding sites define a ligand binding pocket in integrin αIIbβ3.' 275(11) pp. 7795-7802.

Rodgers, R. and Levin, J. (1990) *A critical reappraisal of the bleeding time.* Vol. 16:

Roe, M. T., Armstrong, P. W., Fox, K. A. A., White, H. D., Prabhakaran, D., Goodman, S. G., Cornel, J. H., Bhatt, D. L., Clemmensen, P., Martinez, F., Ardissino, D., Nicolau, J. C., Boden, W. E., Gurbel, P. A., Ruzyllo, W., Dalby, A. J., McGuire, D. K., Leiva-Pons, J. L., Parkhomenko, A., Gottlieb, S., Topacio, G. O., Hamm, C., Pavlides, G., Goudev, A. R., Oto, A., Tseng, C.-D., Merkely, B., Gasparovic, V., Corbalan, R., Cinteză, M., McLendon, R. C., Winters, K. J., Brown, E. B., Lokhnygina, Y., Aylward, P. E., Huber, K., Hochman, J. S., Ohman, E. M. and Investigators, T. A. (2012) 'Prasugrel versus clopidogrel for acute coronary syndromes without revascularization.' *The New England journal of medicine*

Roffi, M., Patrono, C., Collet, J. P., Mueller, C., Valgimigli, M., Andreotti, F., Bax, J. J., Borger, M. A., Brotons, C., Chew, D. P., Gencer, B., Hasenfuss, G., Kjeldsen, K., Lancellotti, P., Landmesser, U., Mehilli, J., Mukherjee, D., Storey, R. F. and Windecker, S. (2016) '2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC).' *Eur Heart J*, 37(3), Jan 14, 2015/09/01, pp. 267-315.

Ronnlund, D., Yang, Y., Blom, H., Auer, G. and Widengren, J. (2012) 'Fluorescence Nanoscopy of Platelets Resolves Platelet-State Specific Storage, Release and Uptake of Proteins, Opening up Future Diagnostic Applications.' *Advanced Healthcare Materials*, 1(6), Nov, pp. 707-713.

Rowland, M., Riegelman, S., Harris, P. A., Sholkoff, S. and Eyring, E. J. N. (1967) 'Kinetics of acetylsalicylic acid disposition in man.' 215(5099) p. 413.

Rubenstein, J., Fischbein, N., Aldape, K., Burton, E. and Shuman, M. (2002) 'Hemorrhage and VEGF expression in a case of primary CNS lymphoma.' *Journal of Neuro-Oncology*, 58(1), May, pp. 53-56.

Saab, Y. B., Zeenny, R. and Ramadan, W. H. (2015) 'Optimizing clopidogrel dose response: a new clinical algorithm comprising CYP2C19 pharmacogenetics and drug interactions.' *Ther Clin Risk Manag*, 11 2015/10/09, pp. 1421-1427.

Sabrkhany, S., Griffioen, A. W. and Egbrink, M. (2011) 'The role of blood platelets in tumor angiogenesis.' *Biochimica Et Biophysica Acta-Reviews on Cancer*, 1815(2), Apr, pp. 189-196.

Sakurai, S., Inoue, A., Koh, C.-S., Owa, M. and Yanagisawa, N. J. V. M. (1997) 'Soluble form of selectins in blood of patients with acute myocardial infarction and coronary intervention.' 2(3) pp. 163-168.

Santos-Martínez, M. J., Prina-Mello, A., Medina, C., & Radomski, M. W. (2011). Analysis of platelet function: role of microfluidics and nanodevices.*Analyst*, *136*(24), 5120-5126.

Sarma, J., Laan, C. A., Alam, S., Jha, A., Fox, K. A. A. and Dransfield, I. (2002) 'Increased platelet binding to circulating monocytes in acute coronary syndromes.' *Circulation*, 105(18) pp. 2166-2171.

Scarborough, R. M., Kleiman, N. S. and Phillips, D. R. J. C. (1999) 'Platelet glycoprotein IIb/IIIa antagonists: what are the relevant issues concerning their pharmacology and clinical use?' 100(4) pp. 437-444.

Scarborough, R. M., Rose, J. W., Hsu, M. A., Phillips, D., Fried, V., Campbell, A., Nannizzi, L. and Charo, I. J. J. o. B. C. (1991) 'Barbourin. A GPIIb-IIIa-specific integrin antagonist from the venom of Sistrurus m. barbouri.' 266(15) pp. 9359-9362.

Schulz, C., Heiss, E., Gaertner, F., Orban, M., Von Bruehl, M.-L., Schramm, P. and Massberg, S. (2009) *Novel Methods for Assessment of Platelet and Leukocyte Function Under Flow - Application of Epifluorescence and Two-Photon Microscopy in a Small Volume Flow Chamber Model.* Vol. 2.

Serebruany, V. L., Sibbing, D. and DiNicolantonio, J. J. J. C. (2014) 'Dyspnea and reversibility of antiplatelet agents: ticagrelor, elinogrel, cangrelor, and beyond.' 127(1) pp. 20-24.

Serebruany, V. L., Steinhubl, S. R., Berger, P. B., Malinin, A. I., Baggish, J. S., Bhatt, D. L. and Topol, E. J. J. T. A. j. o. c. (2005) 'Analysis of risk of bleeding complications after different doses of aspirin in 192,036 patients enrolled in 31 randomized controlled trials.' 95(10) pp. 1218-1222.

Serebruany, V. L., Malinin, A. I., Jerome, S. D., Lowry, D. R., Morgan, A. W., Sane, D. C., Tanguay, J.-F., Steinhubl, S. R. and O'Connor, C. M. (2003) 'Effects of clopidogrel and aspirin combination versus aspirin alone on platelet aggregation and major receptor expression in patients with heart failure: the Plavix Use for Treatment Of Congestive Heart Failure (PLUTO-CHF) trial.' *American Heart Journal*, 146(4) pp. 713-720.

Shankar, H., Kahner, B. N., Prabhakar, J., Lakhani, P., Kim, S. and Kunapuli, S. P. J. B. (2006) 'G-protein–gated inwardly rectifying potassium channels regulate ADP-induced cPLA2 activity in platelets through Src family kinases.' 108(9) pp. 3027-3034.

Shankar, H., Murugappan, S., Kim, S., Jin, J., Ding, Z., Wickman, K. and Kunapuli, S. P. (2004) 'Role of G protein-gated inwardly rectifying potassium channels in P2Y12 receptor-mediated platelet functional responses.' *Blood*, 104(5) pp. 1335-1343.

Shattil, S. J. and Newman, P. J. J. B. (2004) 'Integrins: dynamic scaffolds for adhesion and signaling in platelets.' 104(6) pp. 1606-1615.

Shattil, S. J., Hoxie, J. A., Cunningham, M. and Brass, L. F. (1985) 'Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation.' *Journal of Biological Chemistry*, 260(20) p. 11107.

Shen, J. and DiCorleto, P. E. (2008) 'ADP Stimulates Human Endothelial Cell Migration via P2Y1 Nucleotide Receptor-Mediated Mitogen-Activated Protein Kinase Pathways.' *Circulation Research*, 102(4) pp. 448-456.

Shinoda, Y., Yasutaka, S., Michio, K., Eiseki, U. and Hiroki, A. (2016) 'Potential drug interaction between paclitaxel and clopidogrel.' *Biomedical reports*, 5(1) pp. 141-145.

Siegel-Axel, D. I. and Gawaz, M. (2007) *Platelets and endothelial cells.* Vol. 33: Copyright© 2007 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA.

Smith, W. L. J. A. J. o. P.-R. P. (1992) 'Prostanoid biosynthesis and mechanisms of action.' 263(2) pp. F181-F191.

Smyth, S. S., McEver, R. P., Weyrich, A. S., Morrell, C. N., Hoffman, M. R., Arepally, G. M., French, P. A., Dauerman, H. L., Becker, R. C. and Platelet Colloquium, P. (2009) 'Platelet functions beyond hemostasis.' *Journal of Thrombosis and Haemostasis*, 7(11) pp. 1759-1766.

Soslau, G., Morgan, D. A., Jaffe, J. S., Brodsky, I. and Wang, Y. (1997) 'Cytokine mRNA expression in human platelets and a megakaryocytic cell line and cytokine modulation of platelet function.' *Cytokine*, 9(6), Jun, pp. 405-411.

Stakos, D. A., Gatsiou, A., Stamatelopoulos, K., Tselepis, A. D. and Stellos, K. (2013) 'Platelet microRNAs: From platelet biology to possible disease biomarkers and therapeutic targets.' *Platelets*, 24(8) pp. 579-589.

Stalker, T. J., Newman, D. K., Ma, P., Wannemacher, K. M. and Brass, L. F. *Platelet signaling.* Vol. 210. Germany, 2012.

Stassen, J. M., Arnout, J. and Deckmyn, H. (2004) 'The hemostatic system.' *Current Medicinal Chemistry*, 11(17) pp. 2245-2260.

Steinhubl, S. R., Tan, W. A., Foody, J. M., Topol, E. J. and Jama, E. I. J. (1999) 'Incidence and clinical course of thrombotic thrombocytopenic purpura due to ticlopidine following coronary stenting.' 281(9) pp. 806-810.

Storey, R. F., Oldroyd, K. G. and Wilcox, R. G. J. T. a. h. (2001) 'Open multicentre study of the P2T receptor antagonist AR-C69931MX assessing safety, tolerability and activity in patients with acute coronary syndromes.' 85(03) pp. 401-407.

Storey, R. F., Angiolillo, D. J., Patil, S. B., Desai, B., Ecob, R., Husted, S., Emanuelsson, H., Cannon, C. P., Becker, R. C. and Wallentin, L. J. J. o. t. A. C. o. C. (2010) 'Inhibitory effects of ticagrelor compared with clopidogrel on platelet function in patients with acute coronary syndromes: the PLATO (PLATelet inhibition and patient Outcomes) PLATELET substudy.' 56(18) pp. 1456-1462.

Sugidachi, A., Ogawa, T., Kurihara, A., Hagihara, K., Jakubowski, J., Hashimoto, M., Niitsu, Y. and Asai, F. J. J. o. T. a. H. (2007) 'The greater in vivo antiplatelet effects of prasugrel as compared to clopidogrel reflect more efficient generation of its active metabolite with similar antiplatelet activity to that of clopidogrel's active metabolite.' 5(7) pp. 1545-1551.

Szumilo, M. and Rahden-Staron, I. (2008) '[Phosphoinositide-specific phospholipase C in mammalian cells: structure, properties, and function].' *Postepy Hig Med Dosw (Online)*, 62, Feb 12, 2008/02/20, pp. 47-54.

Terrisse, A., Puech, N., Allart, S., Gourdy, P., Xuereb, J., Payrastre, B. and Sie, P. (2010) 'Internalization of microparticles by endothelial cells promotes platelet/endothelial cell interaction under flow.' *Journal of Thrombosis and Haemostasis*, 8(12) pp. 2810-2819.

Thomas, D. W., Mannon, R. B., Mannon, P. J., Latour, A., Oliver, J. A., Hoffman, M., Smithies, O., Koller, B. H. and Coffman, T. M. (1998) 'Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2.' *The Journal of clinical investigation*, 102(11) pp. 1994-2001.

Thon, J. N., Peters, C. G., Machlus, K. R., Aslam, R., Rowley, J., Macleod, H., Devine, M. T., Fuchs, T. A., Weyrich, A. S., Semple, J. W., Flaumenhaft, R. and Italiano, J. E. (2012) 'T granules in human platelets function in TLR9 organization and signaling.' *Journal of Cell Biology*, 198(4), Aug, pp. 561-574.

Topol, E. J. and Lancet, E. I. J. T. (1998) 'Randomised placebo-controlled and balloon-angioplasty-controlled trial to assess safety of coronary stenting with use of platelet glycoprotein-IIb/IIIa blockade.' 352(9122) pp. 87-92.

Topol, E. J., Byzova, T. V. and Plow, E. F. J. T. L. (1999) 'Platelet GPIIb-IIIa blockers.' 353(9148) pp. 227-231.

Unsworth, A. J., Smith, H., Gissen, P., Watson, S. P. and Pears, C. J. (2011) 'Submaximal inhibition of protein kinase C restores ADP-induced dense granule secretion in platelets in the presence of Ca2.' *The Journal of biological chemistry*, 286(24) pp. 21073-21082.

Valenti, R., Marcucci, R., Comito, V., Marrani, M., Cantini, G., Migliorini, A., Parodi, G., Gensini, G. F., Abbate, R. and Antoniucci, D. (2015) 'Prasugrel in Clopidogrel Nonresponders Undergoing Percutaneous Coronary Intervention: The RECLOSE-3 Study (REsponsiveness to CLOpidogrel and StEnt Thrombosis).' *JACC. Cardiovascular interventions*

Van Giezen, J. and Humphries, R. G. (2005) *Preclinical and clinical studies with selective reversible direct P2Y12 antagonists.* Vol. 31: Copyright© 2005 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA.

van Hinsbergh, V. W. J. E. J. o. O. and Biology, G. a. R. (2001) 'The endothelium: vascular control of haemostasis.' 95(2) pp. 198-201.

Varki, A. (2007) 'Trousseau's syndrome: Multiple definitions and multiple mechanisms.' *Blood*, 110(6), Sep, pp. 1723-1729.

Varon, D. and Shai, E. (2009) 'Role of platelet-derived microparticles in angiogenesis and tumor progression.' *Discovery medicine*, 8(43), 2009-Dec, pp. 237-241.

Verhamme, P. and Hoylaerts, M. J. A. C. B. (2006) 'The pivotal role of the endothelium in haemostasis and thrombosis.' 61(5) pp. 213-219.

Virmani, R., Burke, A. P. and Farb, A. (1999) 'Plaque rupture and plaque erosion.' *Thromb Haemost*, 82 Suppl 1, Sep, 2000/03/01, pp. 1-3.

Vistoli, G., Brizzolari, A., Faioni, E., Razzari, C. and Santaniello, E. (2014) 'Naturally occurring N6-substituted adenosines (cytokinin ribosides) are in vitro inhibitors of platelet aggregation: An in silico evaluation of their interaction with the P2Y12 receptor.' *Bioorganic & Medicinal Chemistry Letters*, 24(24) pp. 5652-5655.

von Beckerath, N. (2008) 'Assessment of ADP-induced platelet aggregation with light transmission aggregometry and multiple electrode platelet aggregometry before and after clopidogrel treatment.'

Wallentin, L., Becker, R. C., Budaj, A., Cannon, C. P., Emanuelsson, H., Held, C., Horrow, J., Husted, S., James, S., Katus, H., Mahaffey, K. W., Scirica, B. M., Skene, A., Steg, P. G., Storey, R. F., Harrington, R. A. and Investigators, P. (2009) 'Ticagrelor versus clopidogrel in patients with acute coronary syndromes.' *New England Journal of Medicine*, 361(11) pp. 1045-1057.

Wallentin, L. C. J. J. o. t. A. C. o. C. (1991) 'Aspirin (75 mg/day) after an episode of unstable coronary artery disease: long-term effects on the risk for myocardial infarction, occurrence of severe angina and the need for revascularization.' 18(7) pp. 1587-1593.

Wang, D., Feng, J., Wen, R., Marine, J. C., Sangster, M. Y., Parganas, E., Hoffmeyer, A., Jackson, C. W., Cleveland, J. L., Murray, P. J. and Ihle, J. N. (2000) 'Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors.' *Immunity*, 13(1) p. 25.

Ware, J., Corken, A. and Khetpal, R. (2013) 'Platelet function beyond hemostasis and thrombosis.' *Current Opinion in Hematology*, 20(5), Sep, pp. 451-456.

Warner, T. D., Armstrong, P. C., Curzen, N. P. and Mitchell, J. A. J. H. (2010) 'Dual antiplatelet therapy in cardiovascular disease: does aspirin increase clinical risk in the presence of potent P2Y12 receptor antagonists?' 96(21) pp. 1693-1694.

Watson, S. P., Asazuma, N., Atkinson, B., Berlanga, O., Best, D., Bobe, R., Jarvis, G., Marshall, S., Snell, D., Stafford, M., Tulasne, D., Wilde, J., Wonerow, P. and Frampton, J. (2001) 'The Role of ITAM- and ITIM-coupled Receptors in Platelet Activation by Collagen.' *Thrombosis and Haemostasis*, 86(1) pp. 276-288.

Wihlborg, A.-K., Wang, L., Braun, O. O., Eyjolfsson, A., Gustafsson, R., Gudbjartsson, T. and Erlinge, D. J. A., thrombosis, and vascular biology. (2004) 'ADP receptor P2Y12 is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels.' 24(10) pp. 1810-1815.

Wijeyeratne, Y. D. and Heptinstall, S. J. B. j. o. c. p. (2011) 'Anti-platelet therapy: ADP receptor antagonists.' 72(4) pp. 647-657.

Williams, R. L. (1999) 'Mammalian phosphoinositide-specific phospholipase C.' *Biochimica et biophysica acta*, 1441(2-3) p. 255.

Wiviott, S. D., Braunwald, E., McCabe, C. H., Montalescot, G., Ruzyllo, W., Gottlieb, S., Neumann, F.-J., Ardissino, D., De Servi, S. and Murphy, S. A. J. N. E. J. o. M. (2007) 'Prasugrel versus clopidogrel in patients with acute coronary syndromes.' 357(20) pp. 2001-2015.

Wiviott, S. D., Antman, E. M., Gibson, C. M., Montalescot, G., Riesmeyer, J., Weerakkody, G., Winters, K. J., Warmke, J. W., McCabe, C. H., Braunwald, E. and Investigators, T.-T. (2006) 'Evaluation of prasugrel compared with clopidogrel in patients with acute coronary syndromes: design and rationale for the TRial to assess Improvement in Therapeutic Outcomes by optimizing platelet InhibitioN with prasugrel Thrombolysis In Myocardial Infarction 38 (TRITON-TIMI 38).' *American Heart Journal*, 152(4) pp. 627-635.

Wiviott, S. D., Braunwald, E., McCabe, C. H., Montalescot, G., Ruzyllo, W., Gottlieb, S., Neumann, F.-J., Ardissino, D., De Servi, S., Murphy, S. A., Riesmeyer, J., Weerakkody, G., Gibson, C. M., Antman, E. M. and Investigators, T.-T. (2007) 'Prasugrel versus clopidogrel in patients with acute coronary syndromes.' *The New England journal of medicine*

Wu, M., KK and Thiagarajan, M., P %J Annual review of medicine. (1996) 'Role of endothelium in thrombosis and hemostasis.' 47(1) pp. 315-331.

Xiang, B., Zhang, G., Liu, J., Morris, A. J., Smyth, S. S., Gartner, T. K. and Li, Z. J. J. o. T. a. H. (2010) 'A Gi‐independent mechanism mediating Akt phosphorylation in platelets.' 8(9) pp. 2032-2041.

Xiao, T., Takagi, J., Coller, B. S., Wang, J.-H. and Springer, T. A. J. N. (2004) 'Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics.' 432(7013) p. 59.

Y.A, N. and A.V, M. (2003) 'Role of Glycoprotein IIb-IIIa (alphaIIbbeta3-integrin) in Stimulation of Secretion from Platelet Granules.' *Biochemistry (Moscow)*, 68(2) pp. 209-216.

Yacoub, D., Théorêt, J.-F., Villeneuve, L., Abou-Saleh, H., Mourad, W., Allen, B. G. and Merhi, Y. J. J. o. B. C. (2006) 'Essential role of protein kinase Cδ in platelet signaling, αIIbβ3 activation, and thromboxane A2 release.
' 281(40) pp. 30024-30035.

Yang, X., Sun, L., Ghosh, S. and Rao, A. K. (1996) 'Human platelet signaling defect characterized by impaired production of inositol-1,4,5-triphosphate and phosphatidic acid and diminished Pleckstrin phosphorylation: evidence for defective phospholipase C activation.' *Blood*, 88(5) p. 1676.

Yen, T.-L., Lu, W.-J., Lien, L.-M., Thomas, P. A., Lee, T.-Y., Chiu, H.-C., Sheu, J.-R. and Lin, K.-H. (2014) 'Amarogentin, a secoiridoid glycoside, abrogates platelet activation through PLC γ 2-PKC and MAPK pathways.' *BioMed research international*

Zetterberg, F. and Svensson, P. (2016) 'State of affairs: Design and structure-activity relationships of reversible P2Y12 receptor antagonists.' *Bioorg Med Chem Lett*, 26(12), Jun 15, 2016/05/03, pp. 2739-2754.

Zhang, F., Yu, W., Hargrove, J. L., Greenspan, P., Dean, R. G., Taylor, E. W. and Hartle, D. K. J. A. (2002) 'Inhibition of TNF-α induced ICAM-1, VCAM-1 and E-selectin expression by selenium.' 161(2) pp. 381-386.

Zhang, Q., Liao, X. and Wu, F. (2017) 'The naphthoquinone plumbagin suppresses ADP-induced rat platelet aggregation through P2Y1-PLC signaling pathway.' *Pakistan journal of pharmaceutical sciences*, 30(2(Suppl.)) p. 573.